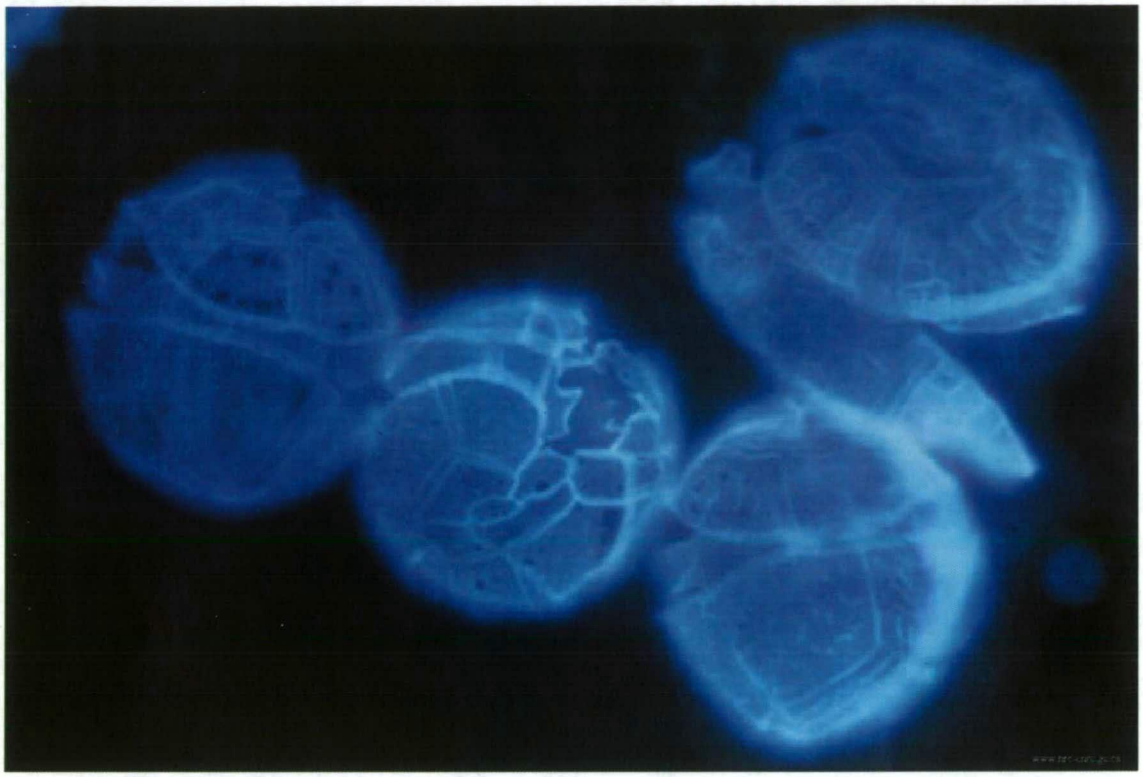
Treatment options to mitigate transport of harmful algal species and pathogens via ships' ballast water and shellfish translocation



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July, 2009

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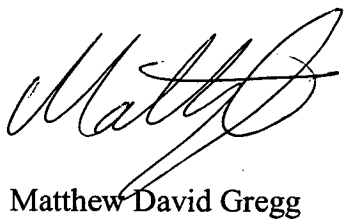

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I would like to dedicate this thesis to my mother, Dianne

Abstract

The worldwide transfer of non-indigenous invasive aquatic organisms via ships' ballast water and shellfish translocation has been widely shown to cause significant ecological, economic and human health impacts. In 2004 the International Maritime Organisation (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments. This legislation (still to be ratified) requires all ships to introduce by 2016 approved systems capable of treating ballast water to strict microbial standards. The latter has reinvigorated interest in the application of chemical biocides, because mechanical separation and physical treatment are unable to kill bacteria. The present work examined the effectiveness of several proposed chemical ballast water treatment options using toxic dinoflagellate resting cysts, vegetative marine microalgae and bacteria as model organisms.

The chemicals tested included the ballast water biocides Peraclean® Ocean and SeaKleen®, the chlorine dioxide biocide Vibrex® and the hull antifouling agent Econea®. All biocide tests were conducted using filtered seawater (28 ‰) and natural estuarine water ranging in salinity from 23.7-28.6‰. Peraclean® Ocean was biodegradable within 2-6 weeks, could effectively eliminate vegetative microalgae at 100 ppm, inactivated resting cysts of marine dinoflagellates (*Gymnodinium catenatum, Alexandrium catenella, A. pseudogonyaulax, Protoceratium reticulatum*) at 200-2000 ppm, and could control bacterial growth of *Escherichia coli, Staphylococcus aureus, Listeria innocua* and *Vibrio alginolyticus* at 125-250 ppm. SeaKleen® eliminated vegetative microalgae at 2 ppm and could control dinoflagellate cysts at 6-20 ppm but displayed poor bactericidal properties (100-200 ppm required) and poor biodegradability. The burial of dinoflagellate cysts in 0.5 and 1 mm of ballast tank sediment severely reduced the effectiveness of Peraclean® Ocean and SeaKleen®. Vibrex® is not a suitable treatment option due to the need for hydrochloric acid as an activator, however it was found to be the most effective against bacteria (complete inhibition at 15 ppm) indicating that onboard chlorine dioxide generators may provide an effective bacterial treatment. Econea® controlled vegetative microalgae at 0.5 ppm but failed to inactivate *G. catenatum* cysts even at a concentration of 1000 ppm, suggesting that the product lacks the penetrability

required to infiltrate the walls of dinoflagellate cysts. The applicability of ballast water biocides is limited by factors such as cost, biological effectiveness, reduced efficacy in the presence of sediments and lower water temperatures (6°C compared to 17°C), and possible residual toxicity of the discharged ballast water.

Translocation of bivalve shellfish for outgrowing purposes or to establish new shellfisheries also poses a significant risk for transport of harmful algae. This study showed that viable microalgal cells of *A. catenella, Cryptoperidiniopsis brodyi, G. catenatum, Karenia papilionacea, Kryptoperidinium foliaceum, Pfiesteria shumwayae* and cysts of *G. catenatum* can readily pass through the digestive tract of adult Pacific oysters and blue mussels. Several treatment options currently used by shellfish farmers for controlling fouling and pathogens were tested against Pacific oysters. Vegetative cells of *A. catenella, G. catenatum* and *K. foliaceum* could not be eliminated from the digestive tract of Pacific oysters following 24 h immersion in freshwater or by 48 h exposure to hydrogen peroxide (400-600 ppm) or chlorine dioxide (40-60 ppm). Depuration in filtered seawater for 7 days was identified as the only effective treatment option. Recommendations for future research, along with potential alternative treatment options, are identified and discussed.

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Chapter 1

General Introduction

1.1 Introduction

The worldwide transfer and introduction of non-indigenous aquatic organisms can lead to dramatic alterations of marine and freshwater environments. Although some aquatic invasions result from natural dispersal mechanisms, the majority appear to be caused by human activities. Anthropogenic introductions of non-indigenous aquatic organisms have been occurring on a global scale for hundreds, if not thousands of years. Unfortunately, the rate of new invasions appears to be increasing and is now widely recognised as one of the main threats to the world's oceans and aquatic ecosystems. Evidence is accumulating that ecosystems disturbed by pollution or climate change are especially prone to biological invasions (Stachowicz *et al.*, 2002).

There are several means by which species may become introduced. Ballast water transport and hull-fouling transfer by commercial ships have been suggested to be the two primary vectors for aquatic introductions (Minchin and Gollasch, 2002). Other mechanisms include aquaculture, seafood, bait and aquarium enterprises; recreational vessels; seaplanes; marine debris; migrating birds and ocean current movements (Carlton, 1985; Carlton and Geller, 1993; Darrigan, 2002; Barnes and Milner, 2005; Weigle *et al.*, 2005). Of these non-shipping mechanisms, the aquaculture industry has played a substantial role in the global transfer of non-indigenous marine organisms, particularly with regard to the translocation of bivalve shellfish (Minchin and Gollasch, 2002).

This thesis focuses on the anthropogenic introduction of non-indigenous species via ships' ballast water and associated with the translocation of bivalve shellfish for aquaculture. The thesis is presented as a general introduction (**Chapter 1**), a series of stand-alone chapters on aspects of ballast water treatment (**Chapters 2-5**), a comprehensive review on recent progress in the development of treatment options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water (**Chapter 6**) and two chapters on the transfer of harmful microalgae via bivalve shellfish translocation (**Chapters 7 and 8**).

1.2 Ships' ballast water as a vector for the introduction of aquatic species

Ballast water is routinely taken onboard ships to maintain safety and stability at sea. Correct ballasting reduces stresses on the hull of the ship, provides stability, aids propulsion and manoeuvrability, and compensates for weight lost from unloading cargo and from fuel consumption (National Research Council, 1996). Today, ballast water is gravity fed or pumped into specially designed ballast tanks or cargo holds. During this process, any organisms and sediment that are suspended in water column are also taken on board.

On a daily basis, it is estimated that over 7,000 different species are being transported around the globe in ships ballast water (Carlton, 1999). Most organisms taken onboard during ballast intake do not survive the voyage or fail to establish viable populations in the receiving region once discharged, however, in an increasing number of documented cases, some organisms do survive the voyage, manage to establish in their new environment and ultimately dominate the ecosystem resulting in vast ecological and environmental impacts. In the Great Lakes of North America, for example, 43 species have been introduced and established since 1959, of which an estimated 67% have been due to ballast water transport (Grigorovich *et al.,* 2003). Historically, the most aggressive ballast water invader has been the European zebra mussel *Dreissena polymorpha*. This organism was introduced into the Great Lakes in the late 1980's and since has dominated the ecosystem causing significant economic loss through the fouling of industrial water intake pipes and municipal structures (Cohen and Carlton, 1998). The economic impact of the zebra mussel is estimated to be as high as US$ 1 billion per year (Pimentel, 2005) and eradication appears impossible, as the problem has now spread to 23 U.S. states and 2 Canadian provinces (Bossenbroek *et al.,* 2007). Other examples of ballast water introductions that have resulted in extensive environmental and economic impacts include the golden mussel *Limnoperna fortunei* in South America (Karatayev *et al.,* 2006; Burlakova, 2007), the Chinese mitten crab *Eriocheir sinensis* in North America and Europe (Ruiz *et al.,* 1997; Cohen and Carlton, 1998), the Atlantic comb jelly *Mnemiopsis leidyi* in the Black, Azov and Caspian seas (Ruiz *et al.,* 1997), the Northern Pacific seastar *Asterias amurensis* and the Japanese kelp *Undaria*

pinnatifida in Tasmania, Australia and the European Green crab *Carcinus maenas* in North America, Australia and Japan (Bax *et al.*, 2003).

Ballast-mediated introductions can also lead to ecological problems that can adversely affect human health. For example, ballast water has been implicated in the global dissemination of pathogenic bacteria and viruses (including *Vibrio cholerae* and *Escherichia coli*) (McCarthy and Khambaty, 1994; Ruiz *et al.*, 2000; Dobbs *et al.*, 2003; Ivanov *et al.*, 2003; Burkholder *et al.*, 2007), and has been shown to transport viable phytoplankton species responsible for human illnesses such as paralytic shellfish poisoning, amnesic shellfish poisoning, diarrhetic poisoning and ciguatera fish poisoning (Hallegraeff and Bolch, 1992; Kelly, 1993; Hallegraeff, 1998; Forbes and Hallegraeff, 2001; Hamer *et al.*, 2000, 2001).

In recognition of the potentially devastating effects from the introduction of invasive species in ships ballast water, the International Maritime Organisation (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments on the 13th of February 2004. One of the main requirements of this legislation is the phased implementation of a ballast water discharge standard, whereby, depending on construction date and ballast water capacity, ships must conduct ballast water exchange as an interim strategy and eventually use an approved ballast water treatment system capable of treating ballast water to the following standard:

Regulation D-2 of the Convention specifies that ships meeting the requirements of the Convention must discharge:

- less than 10 viable organisms per cubic meter greater than or equal to 50 micrometers in minimum dimension, and
- less than 10 viable organisms per millilitre less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension, and
- less than the following concentrations of indicator microbes, as a human health standard:

- Toxigenic *Vibrio cholerae* (serotypes 01 and 0139) with less than 1 Colony Forming Unit (cfu) per 100 millilitres or less than 1 cfu per 1 gram (wet weight) of zooplankton samples,
- *Escherichia coli* less than 250 cfu per 100 millilitres, and
- Intestinal *Enterococci* less than 100 cfu per 100 millilitres.

The convention will enter into force 12 months after ratification by 30 countries representing 35% of the world's commercial shipping tonnage (IMO, 2004). As of September 30, 2008, only 16 countries have signed the Convention. The implementation of the IMO Convention was originally set to begin in 2009, however due to pressure from the shipping industry and the refusal of some countries to ratify the convention due to the limited availability of effective treatment systems, the IMO has delayed the initial enforcement date by up to three years allowing for the further development of treatment technologies. The adoption of the new Convention coupled with the limited effectiveness and operational limitations of ballast water exchange has led to significant financial investment in the research and development of shipboard ballast water treatment technologies. Some estimates forecast that US$10-15 billion will be spent on ballast water treatment research during the next decade. A wide variety of treatment options have been proposed for ballast water treatment but it is increasingly believed that the use of chemical biocides or active substances will be required to comply with the microbial component of the IMO ballast water discharge standard.

This thesis examines the effectiveness of several proposed chemical ballast water treatment options using toxic dinoflagellate cysts, vegetative marine microalgae and bacteria as model organisms. Toxic dinoflagellates are of particular concern in relation to ballast-mediated dispersal due to the formation of resistant sexual cysts (Figure 1), which can remain viable in sediment for decades (Hallegraeff and Bolch, 1992; Lewis *et al.*, 1999).

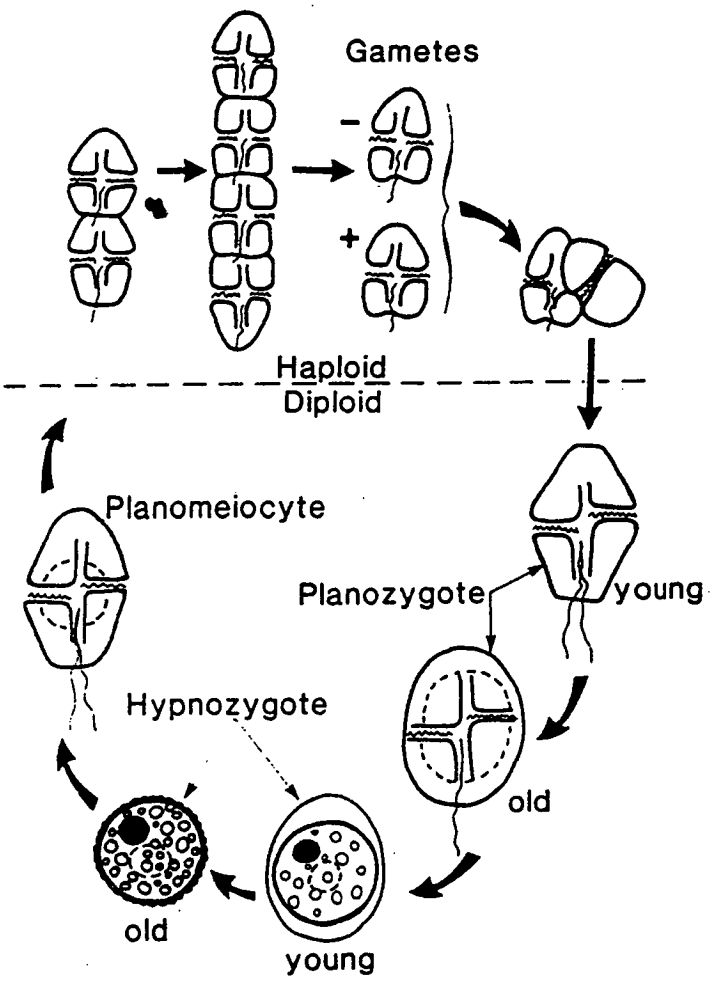

Fig. 1. Lifecycle of a typical cyst forming toxic dinoflagellate *Gymnodinium catenatum* illustrating the process of *zygotic meiosis* (after Blackburn *et al.* 1989).

Dinoflagellate cysts are one of the most frequently found organisms in ships' ballast water and tank sediments and provide a good model organism for the assessment of ballast water treatment options as they are extremely robust and have been demonstrated to be resistant to several sterilisation techniques including UV irradiation and chlorination. Therefore, it can be argued that a treatment capable of killing dinoflagellate cysts will likely kill a wide range of other target organisms that occur in ballast water and sediments. **Chapter 2** examines the efficacy of the commercial ballast water biocides Peraclean® Ocean and SeaKleen®, and the chlorine dioxide biocide Vibrex® for controlling dinoflagellate cysts, marine microalgae and bacteria and **Chapter 3** tests the effectiveness of the hull anti-foulant ECONEA® Technical and suitable co-biocides against vegetative microalgae and dinoflagellate cysts. The degradability of Peraclean® Ocean, SeaKleen® and ECONEA® Technical

was assessed as any potential chemical treatment must degrade to a non-toxic level prior to discharge to avoid negative impacts on the marine environment. Ballast tanks can also accumulate significant amounts of sediment providing an additional habitat for a variety of resistant organisms including resting stages of phytoplankton and zooplankton and microbial pathogens, therefore, a successful chemical treatment must also be able to eliminate organisms that reside in ballast tank sediments. **Chapter 4** assesses the ability of Peraclean® Ocean and SeaKleen® to inactivate dinoflagellate cysts buried in ballast tanks sediment.

To date, the use of dinoflagellate cysts for assessing the efficacy of ballast water treatment options has been limited due to the time required to determine cyst viability following exposure to potential treatment options. Conventional methods for determining cyst viability require careful microscopic observations of cyst germination for a period of up to several months. **Chapter 5** examines the reliability of a more rapid technique for determining cyst germination using the vital stain Sytox® Green.

Apart from chemical treatments, other technologies currently under commercial development include mechanical separation devices, heat treatment, UV irradiation, ultrasound, de-oxygenation and treatment systems that incorporate multiple technologies. **Chapter 6** provides a detailed up-to-date review of progress in the development of treatment options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water and sediments.

1.3 Bivalve shellfish transfers as a vector for the introduction of invasive aquatic organisms

Large quantities of shellfish are often transferred from one water body to another to restock or establish new aquaculture fisheries (Wolff and Reise, 2002). Live shellfish are known to harbour a variety of organisms and have been documented or suggested to be a possible vector for the introduction of parasites and pathogens, harmful microalgal and macroalgal species, and various invertebrates (Humphrey, 1988; Chew, 1990; Minchin, 1996; Wolff and Reise, 2002; Mineur *et al.*, 2007). In European aquatic environments, the translocation of shellfish for aquaculture is

considered to be the dominant source of non-indigenous species introductions, with estimates suggesting that 44% of the introductions may be attributed to shellfish movements (Wallentinus, 2002). In many instances, little has been done to avoid the transfer of non-indigenous organisms associated with the introduction and translocation of live shellfish. For example, an examination of oyster spat exported from France to Ireland in 1993 revealed the presence of a wide variety of organisms (Minchin *et al.,* 1993) including 67 phytoplankton species and 15 types of dinoflagellate cysts, even though the shellfish were deemed to be free of other organisms (O'Mahony, 1993).

It is widely acknowledged that translocated shellfish are potential carriers of phytoplankton between aquaculture sites. Several researchers have shown that harmful phytoplankton species can remain intact in the digestive tracts of certain molluscs and may recover into viable populations once excreted in faecal material (e.g. Laabir and Gentien, 1999; Laabir *et al.,* 2007; Hégaret *et al.,* 2008); however, studies of this kind have never been completed in Australia. In **Chapter 7**, we examined whether viable microalgal cells of the dinoflagellates *Alexandrium catenella, Cryptoperidiniopsis brodyi, Gymnodinium catenatum, Karenia papilionacea, Krytoperidinium foliaceum, Pfiesteria shumwayae* and resting cysts of *G. catenatum* can pass intact through the digestive tract of two widely exploited bivalve species in Australia, the non-indigenous Pacific oyster (*Crassostrea gigas*) and the native blue mussel (*Mytilus galloprovincialis*), and whether intact cells excreted in the bivalve faeces can recover into viable populations.

Several elimination, or preventative, treatment techniques are currently used to reduce the abundance of organisms transported with live shellfish transfers. These include immersion in freshwater, hot water or brine (Mineur *et al.,* 2007); periodical drying; and seawater depuration systems (Richards, 1988). At present, shellfish farmers are also considering using chemical treatments, such as chlorine, however, these treatments focus solely on either eliminating organisms that occur on the outer shell surface of the shellfish or reducing the levels of bioconcentrated bacteria and viruses within the shellfish (Richards, 1988). Given that the introduction of harmful microalgal species into sensitive areas used for aquaculture can result in vast ecological problems, economic loss, and even human health problems due to

exposure to toxins from microalgae through consumption of contaminated shellfish (Hallegraeff, 1992; Burkholder, 1998), it is surprising so little attention has been paid to eliminating, or minimising, phytoplankton associated with live shellfish transfers. **Chapter 8** examines the effectiveness of several potential treatment options for eliminating ingested marine microalgae in the Pacific oyster as the extent to which the current treatment techniques used by shellfish farmers may reduce this risk is largely unknown.

Eliminating the risk of transferring and introducing harmful algal species and pathogns via the discharge of ships' ballast water and the translocation of bivalve shellfish for aquaculture represents a significant challenge for the scientific community. At present, no treatment option or management strategy is available to completely eliminate this threat. Recommendations for future research, along with potential alternative treatment options, are identified and discussed.

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Chapter 2

Efficacy of three commercially available ballast water biocides against vegetative microalgae, dinoflagellate cysts and bacteria

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Chapter 3

Testing the effectiveness of ECONEA® Technical and suitable co-biocides against vegetative microalgae and dinoflagellate cysts

3.1 Abstract

ECONEA™ is a non-metal biocide initially developed for use as a ship hull antifouling agent. In addition to its application in marine antifouling coatings, current research is evaluating ECONEA™ as a ballast water treatment option. Here, we examine the effectivessness of ECONEA™ and 14 potential co-biocides against vegetative microalgae and dinoflagellate cysts, and assess the biodegradability of ECONEA™, as environmental concerns exist regarding the discharge of treated ballast water. The biocide ECONEA® Technical effectively killed vegetative cells of a broad range of both armoured (cellulose-walled) and unarmoured dinoflagellates and raphidophytes at a concentration of 0.5 ppm. ECONEA® was not as effective at controlling the small haptophyte *Prymnesium parvum* (8 ppm required) and did not inactivate the small cellulose-walled green-flagellate *Tetraselmis suecica* even at 20 ppm. ECONEA® failed to inactivate the sexual resting cysts of the dinoflagellate *Gymnodinium catenatum* at a concentration of 1000 ppm, suggesting that the product lacks the penetrability required to infiltrate the resistant cell walls of dinoflagellate cysts. In order to inactivate dinoflagellate cysts in ships ballast water, ECONEA® therefore would require a co-biocide. Out of the 14 potential co-biocides tested, Natrium PYRION® 40% and PYRION® Disulfide 40% Suspension required the lowest concentrations for the effective control of both vegetative microalgae and *Gymnodinium catenatum* cysts. ECONEA® Technical was biodegradable within 4-6 weeks (initial concentration of 1 ppm) under conditions of 12 h light/12 h dark. Degradation was accelerated in the presence of ballast tank sediments but was significantly reduced in the dark.

Keywords: Ballast water treatment; Chemical biocides; Dinoflagellate cysts

3.2 Introduction

Introductions of non-indigenous species are causing dramatic changes in marine communities and ecosystems worldwide. These introductions derive from a variety of vectors; however, due to the large volumes of water involved and the frequency of use, ships ballast water is widely recognised as a major vector for aquatic invasions. One solution to eliminate the threat of ballast-mediated invasive species involves treating ballast water to exclude, remove or kill these organisms.

The treatment of ships ballast water is one of the most significant challenges facing the maritime industry today. In 2004, a global requirement for ballast water treatment arose when the International Maritime Organisation (IMO) adopted the Convention for the Control and Management of Ships' Ballast Water and Sediments. One of the main requirements of the convention is the phased implementation of a ballast water discharge standard, whereby, depending on construction date and ballast water capacity, ships will be required to use an approved ballast water treatment system capable of treating ballast water to strict microbial standards. The implementation of the IMO convention was originally set to begin in 2009, however due to pressure from the shipping industry and the refusal of some countries to ratify the convention due to the limited availability of effective treatment systems, the IMO has delayed the initial enforcement date by up to three years allowing for the further development of treatment technologies.

A wide variety of physical and chemical treatment options have been considered by the scientific community. Current ballast water treatment research has tended to focus on the use of chemical biocides or active substances. This is due to a number of reasons. Firstly, the inclusion of a microbial standard in the IMO convention tends to favour the use of chemical biocides over many environmentally sound treatment options such as heat treatment and filtration. Another advantage is that the use of chemical biocides or active substances is not limited by the high flow rate of ballast pumps. Potential chemicals must be safe to handle by crew members, must be non-corrosive to the ships pipes and structural components, must eliminate all target ballast water biota (including bacteria) at a cost effective concentration, and must rapidly degrade into environmentally benign by-products because of concerns of

chemical discharge into the environment. Many companies have already produced onboard treatment systems that make use of active substances or chemical biocides. To date, eight of these systems have received basic approval and two have been given final approval from the IMO. The two commercially available treatment systems that have received final approval from the IMO are the SEDNA® Ballast Water Management System (Using Peraclean® Ocean) and the PureBallast System (GESAMP, 2008).

ECONEA™ is a non-metal biocide developed by The Preservation and Material Protection Division of Janssen Pharmaceutica NV for use as a hull antifouling agent. In addition to its application in marine antifouling coatings, current research is evaluating ECONEA™ as a ballast water treatment option. Janssen Pharmaceutica NV indicates several features of the compound that potentially make it an attractive ballast water biocide. It is suggested to be biologically effective at low concentrations; it is claimed to degrade rapidly and does not accumulate in the marine environment; and it is a non-oxidative compound therefore would not pose any corrosion problems. Preliminary investigations into its effectiveness as a ballast water biocide found that it was extremely effective against zooplankton species yet was ineffective against microalgae (Tony Kempen, Janssen Pharmaceutica NV, pers. comm.). The main objective of the present work was to test the effectiveness of ECONEA™ and potential co-biocides against a range of vegetative microalgal species and dinoflagellate cysts of *Gymnodinium catenatum*; and to assess the degradability of ECONEA™, as any potential ballast water biocide must degrade to a concentration low enough to avoid ecological impacts following discharge into receiving waters.

3.3 Materials and methods

Vegetative microalgal cultures

Vegetative cultures of the armoured (cellulose-walled) dinoflagellates *Alexandrium catenella, Protoceratium reticulatum, Scrippsiella trochoidea,* unarmoured dinoflagellates *Gymnodinium catenatum, Karlodinium veneficum, Karenia papilionacea*, the raphidophytes *Chattonella marina* and *Heterosigma akashiwo,* and

the haptophyte *Prymnesium parvum* were grown in 250 ml culture flasks containing 150 ml of GSe medium (Blackburn *et al.*, 1989). The cellulose-walled green flagellate *Tetraselmis suecica* was grown in 250 ml culture flasks containing 150 ml of f/2 medium (Guillard, 1975). Nutrient medium was made with ultra-filtered seawater collected from Bruny Island, Tasmania, Australia. Table 1 summarises isolation details including cell sizes of the microalgal strains used. All cultured microalgal species were obtained from the microalgal culture collection at the School of Plant Science, University of Tasmania. *Alexandrium catenella* and *Gymnodinium catenatum* are paralytic shellfish poison (PSP) producing toxic dinoflagellates, *Karlodinium veneficum, Karenia papilionacea*, *Chattonella marina*, *Heterosigma akashiwo, Prymnesium parvum* are all fish killers, while *Protoceratium reticulatum, Scrippsiella trochoidea* and *Tetraselmis suecica* are non-toxic. All cultures were maintained in a culture room at 17°C under 12 h dark/12 h light. Light was provided at an intensity of 100 μmol quanta $m^{-2}s^{-1}$ by a bank of cool-white fluorescent tubes.

Table 1. Isolation details and characteristics of microalgal strains used

Species	Algal group	Cell size (μm)	Culture code	Date of isolation	Source	Isolated by	Status
Alexandrium catenella	Armoured dinoflagellate (PSP)	25-32	ACSH02	N/A	Sydney Harbour (NSW)	N/A	N/A
Chattonella marina	Raphidophyte (fish-killer)	30-70	CMDE01	N/A	Derwent River (TAS)	M. de Salas	N/A
Gymnodinium catenatum	Unarmoured dinoflagellate (PSP)	27-36	GCDE11	N/A	Derwent River (TAS)	M. de Salas	Unialgal
Gymnodinium catenatum	Unarmoured dinoflagellate (PSP)	27-46	GCTRA01	24/05/2000	Triabunna (TAS)	M. de Salas	Clonal
Heterosigma akashiwo	Raphidophyte (fish-killer)	11-25	HAHB02	N/A	Hidaway Bay (TAS)	M. de Salas	Clonal
Karenia papilionacea	Unarmoured dinoflagellate (fish killer)	18-32	KPPL01	27/05/2003	Port Lincoln (SA)	M. de Salas	Clonal
Karlodinium veneficum	Unarmoured dinoflagellate (fish killer)	8-14	KVSH01	15/05/2002	Sydney Harbour (NSW)	M. de Salas	Clonal
Protoceratium reticulatum	Armoured dinoflagellate	28-53	PTRDE11	1999	Derwent River (TAS)	N. Parker	Clonal
Prymnesium parvum	Haptophyte (fish killer)	8-15	PPSR01	N/A	Serpentine River (WA)	J. Marshall	N/A
Scrippsiella trochoidea	Armoured dinoflagellate	16-36	SCAD01	15/02/2000	Adelaide (SA)	M. de Salas	Unialgal
Tetraselmis suecica	Green flagellate	10-15	TSCS187	N/A	Brest, France	A.Dodson	N/A

Dinoflagellate cyst production

Two ml culture suspensions of compatible sexual mating strains of the dinoflagellate *Gymnodinium catenatum* (strain GCDE11 x GCTRA01) were inoculated into 250 ml screw top beakers containing 200 ml of filtered seawater (28‰ salinity) with 10 ml of GSe medium. The screw top beakers were incubated at 17°C under 12 h dark/12 h light with a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ and were examined at regular intervals for cyst formation. This procedure produced approximately 1500 cysts per beaker within 28 days. Prior to treatment, cysts were stored at 4°C in the dark to prevent premature germination.

Chemical biocides

All chemical biocides were supplied by Janssen PMP, Belgium. For ECONEA™, the stock solution was prepared from a newly opened container of ECONEA® Technical in the form of a wettable powder (Batch no. ZR107894EXA037; Active content: 98.4 wt. %; Janssen PMP, Belgium). Product and company information on the 14 possible co-biocides is summarised in Table 2. All treatment solutions were prepared in filtered seawater (28‰ salinity).

Chemical treatment of vegetative microalgae

One ml of vegetative microalgal cultures (*Gymnodinium catenatum, Alexandrium catenella, Protoceratium reticulatum, Scrippsiella trochoidea, Karlodinium veneficum, Karenia papilionacea, Chattonella marina, Heterosigma akashiwo, Prymnesium parvum, Tetraselmis suecica*) were placed into 24-well flat bottom microplates and various concentrations of ECONEA® Technical were applied. The 14 potential co-biocides were only tested against vegetative *A. catenella*, *P. parvum* and *T. suecica* cells but Rimsulfuron PESTANAL® and Isoxaflutol PESTANAL® were not tested against *P. parvum* and *T. suecica*. Microalgal cultures used were two weeks old and contained approximately 1–2 x 10^6 cells l^{-1} (1–5 x 10^7 cells $l^{-1}$for *P. parvum* and *T. suecica*). Each biocide treatment was replicated three times. Culture plates were sealed with parafilm following the biocide application and placed under a

light intensity of 100 µmol quanta $m^{-2}s^{-1}$ at 17°C with a 12 h dark/12 h light photoperiod. Cell viability was assessed after 48 h by calculating the percentage of non-viable cells for each of the various biocide concentrations and control treatments. Percentage cell viability was determined by counting the number of non-viable cells out of 100 total cells. Motility, flagellar movement, and cell wall and organelle integrity were used as indicators of viability. Viability observations were conducted on a Zeiss inverted microscope (Zeiss Axiovert 25).

Chemical treatment of dinoflagellate cysts

The reliable assessment of dinoflagellate cyst viability was essential to determine lethal concentrations of the chemical biocides tested. The present study used the existence of a healthy swimming planomeiocyte and the incidence of an empty cyst wall as criteria for cyst germination and survival (e.g. Ichikawa *et al.,* 1992; Bolch and Hallegraeff, 1993; Hallegraeff *et al.,* 1997). For the chemical treatments, dinoflagellate cysts were placed into 12-well flat bottom microplates containing 4 ml of GSe medium and various concentrations of the biocides were subsequently applied. Each concentration and control was replicated 3 times. After the application of the biocide, the culture plates were sealed with parafilm, wrapped in aluminium foil and placed under culture conditions at 17°C. After 2 weeks exposure, cysts were removed, washed in sterile GSe medium, and transferred to new 12-well culture plates containing fresh GSe media. The treated cysts were then placed back under culture conditions with a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ at 17°C with a 12h dark/ 12h light photoperiod. Treatments were examined at weekly intervals for cyst germination. Germination observations were conducted on a Zeiss inverted microscope (Zeiss Axiovert 25).

Table 2. Product and Company Information of the 15 biocides tested.

Product name	Product form	Batch number	Composition/Information on ingredients	Active content	Manufacturer
ARQUAD MCB-50	Liquid	654S6005	C12-C16 alkylbennzyldimethylammonium chloride ~50%. Water~48%. Ethylene glycol~2%	50 wt. %	Akno Nobel, Sweden
ECONEA® Technical	Solid	ZR107894EXA037	2-(p-chlorophenyl)-3-cyano-4-bromo-5-trifluoromethyl pyrrole	98.4 wt. %	Janssen Pharmaceutica N.V, Belgium
Hydrogen peroxide 30%	Liquid	612809028	Hydrogen peroxide	30 wt. %	Mallinckrodt Baker B.V., The Netherlands
Intercide OIT	Liquid	Z10192	2-n-Octyl-4-Isothiazolin-3-one	>96 wt. %	Akros Chemicals, England
Isoxaflutol PESTANAL®	Solid	46437	C15H12F3NO4S	n/a	Sigma-Aldrich, Belgium
KATHON™ 886F Biocide	Liquid	1735729	Magnesium nitrate. Mixture of: 5-chloro-2-methly-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (3:1)	14.0 wt. %	Rohm and Haas Company, United Kingdom
LAg 2006 041	Liquid	SID3865	confidential	11.34 wt. %	Janssen Pharmaceutica N.V, Belgium
LAg 2007 030	Liquid	SID4076	confidential	0.05 wt. %	Janssen Pharmaceutica N.V, Belgium
Natrium PYRION® 40%	Liquid	AA270488EXA002	Pyridine-2-thiol 1-oxide, sodium salt	40.7 wt. %	Janssen Pharmaceutica N.V, Belgium
Oxy-PYRION® 98%	Solid	A6010010	2(1H)-Pyridione, 1-hydroxy-	99.4 wt. %	Janssen Pharmaceutica N.V, Belgium
PROTECTNOL BN	Solid	7044R1	bronopol	>99 wt. %	BTC Chemical Distribution Ltd, United Kingdom
PYRION® Disulfide 40% Suspension	Liquid	A6061810	2,2'Dithiobis(pyridine-1-oxide)	41.3 wt. %	Janssen Pharmaceutica N.V, Belgium
Rimsulfuron PESTANAL®	Solid	46097	C14H17N5O7S2	n/a	Sigma-Aldrich, Belgium
R090026	Solid	2001 014/SID1012	confidential	80 wt. %	Janssen Pharmaceutica N.V, Belgium
4, 5-dichloro-N-octyl-4-isothiazolin-3-one	Solid	51226	4, 5-dichloro-N-octyl-4-isothiazolin-3-one	98.5 wt. %	Chemos GmbH, Germany

ECONEA™ *degradation*

The degradability of the ECONEA™ was examined indirectly by testing the effectiveness of the biocide against vegetative microalgae in different seawaters and light conditions over time. Two concentrations of ECONEA® Technical (1 ppm, 10 ppm) were prepared in 200 ml of filtered seawater (28‰ salinity), 200 ml of filtered seawater (28‰ salinity) containing 0.1, 0.5 and 1 g of ballast tank sediment, and 200 ml of natural estuarine water collected from the Derwent (27.9‰) and Huon (25.9‰) Rivers in Southern Tasmania. Ballast tank sediment was collected from the MV Princess Betty (No. 3 Port topside tank) in Geelong, Australia on 26-11-1989 and stored at 4°C in the dark until use. Replicates of each biocide concentration were prepared allowing samples to be stored in the dark and under 12h light/12h dark at 17°C. Trials were conducted in triplicates and the degradability of the biocides was assessed weekly by applying the various ageing concentrations to four separate vegetative microalgal cultures (*G. catenatum, A. catenella, P. reticulatum, C. marina*). This method allowed a comparison of the sensitivity of the individual microalgal species to the ageing biocide concentrations. One ml of each culture was placed into individual wells of 24-well flat bottom microplates and the aging biocide samples were applied. This procedure was repeated weekly for up to 16 weeks. Cell viability was assessed following an exposure period of 48 h by calculating the percentage of non-viable cells for each of the various biocide concentrations and control treatments. Percentage cell viability was determined by counting the number of non-viable cells out of 100 total cells. Viability observations were conducted on a Zeiss inverted microscope (Zeiss Axiovert 25).

3.4 Results

Chemical treatment of vegetative microalgae

ECONEA® Technical was found to be the most effective against vegetative cells of the unarmoured dinoflagellate *G. catenatum* and raphidophyte *C. marina*. No viable cells of either species were found at a concentration of 0.1 ppm following an exposure period of 48 h (Fig. 1A, B). Figure 2 shows vegetative *G. catenatum* cells exposed to 0.5 ppm of ECONEA® Technical. Following treatment, cells quickly lost

motility, cell contents appeared discoloured, and ultimately the cell integrity was compromised and disintegration occurred. Vegetative cells of the dinoflagellates *A. catenella* (armoured), *K. papilionacea* (unarmoured), *P. reticulatum* and *S. trochoidea* (both armoured), could be controlled at a concentration of 0.25 ppm following 48 h exposure to the biocide (Fig. 1C-F). At this concentration (0.25 ppm), 97% of *K. veneficum* and 1% of the *H. akashiwo* were still viable after 48 h exposure, however complete mortality of both species was observed at a concentration of 0.5 ppm (Fig. 1G, H). ECONEA® Technical was considerably less effective against vegetative cells of the small haptophyte *P. parvum* and small green flagellate *T.suecica.* No viable cells of *P. parvum* remained following 48 h at a concentration of 8 ppm (Fig. 1I), and complete control of *T. suecica* was not achieved at a concentration of 20 ppm (Fig. 1J). At 20 ppm, 65% of *T. suecica* cells were still viable. Twenty ppm was the maximum concentration tested in the experiments.

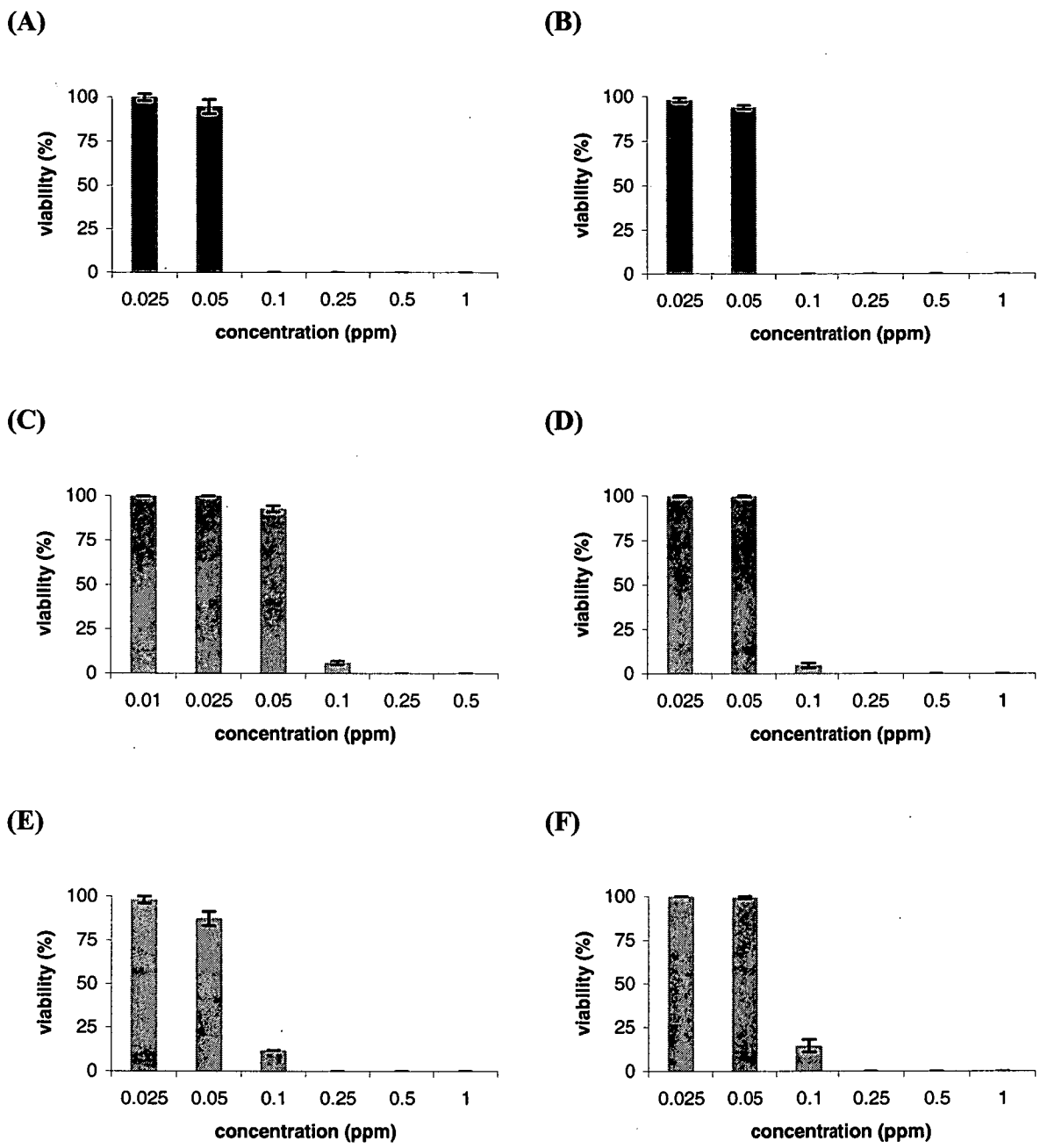
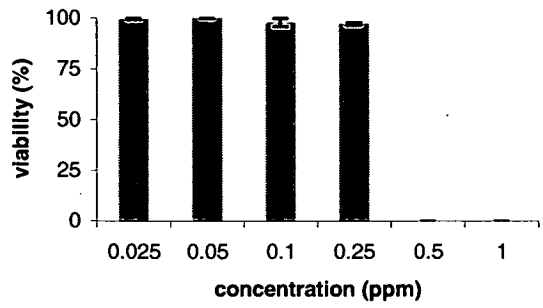
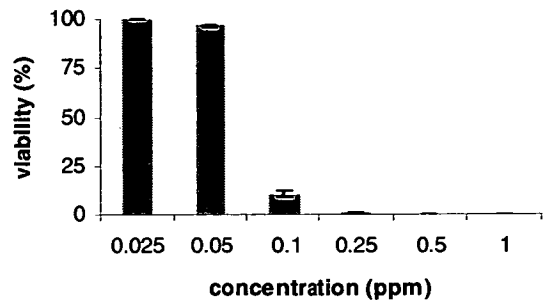


Fig. 1. Effect of varying concentrations of the biocide ECONEA® Technical on the viability of different vegetative microalgal species (48 h exposure). (A) *Gymnodinium catenatum*; (B) *Chattonella marina*; (C) *Alexandrium catenella*; (D) *Karenia papilionacea*; (E) *Protoceratium reticulatum*; (F) *Scrippsiella trochoidea*. Bars indicate standard error.

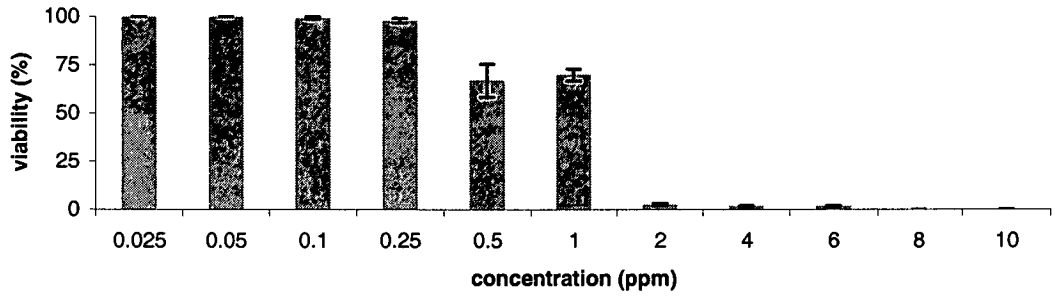
(G)



(H)



(I)



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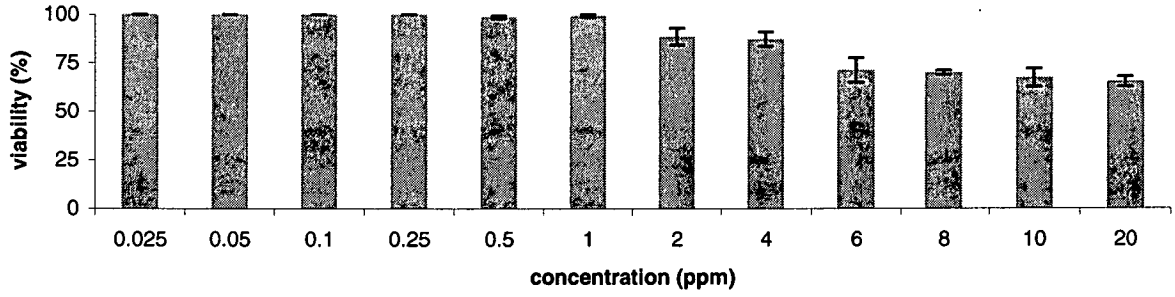


Fig. 1 *cont.*. Effect of varying concentrations of the biocide ECONEA® Technical on the viability of different vegetative microalgal species (48 h exposure). (G) *Karlodinium veneficum*; (H) *Heterosigma akashiwo*; (I) *Prymnesium parvum*; (J) *Tetraselmis suecica.* Bars indicate standard error.

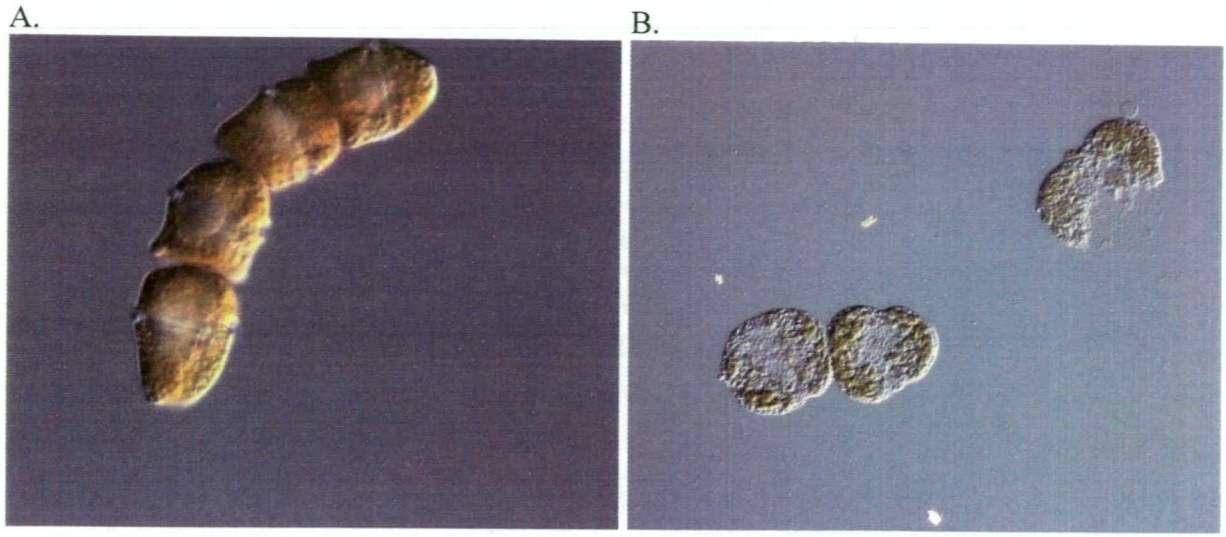


Fig. 2. Morphological changes of vegetative *Gymnodinium catenatum* cells exposed to ECONEA® Technical. (A) Control treatment. (B) 48 h exposure to 0.5 ppm ECONEA® Technical.

The effectiveness of the 14 possible co-biocides against vegetative cells of the dinoflagellate *A. catenella* is presented in Figure 3. The concentrations required to eliminate viable cells varied considerably between products. For example, the biocides Natrium PYRION® 40% and PYRION® Disulfide 40% Suspension were extremely effective at concentrations of 0.025 and 0.1 ppm, respectively, following an exposure period of 48 h (Fig. 3A, B). On the contrary, complete control was not achieved in the Rimsulfuron PESTANAL® or Isoxaflutol PESTANAL® treatments at the maximum tested concentration of 1000 ppm and the biocide LAg 2007 030 required 5000 ppm to completely eliminate viable cells (Fig. 3.C, D, F). The KATHON™ 886F, ARQUAD MCB-50, PROTECTNOL BN, R090026, LAg 2006 041 and Intercide OIT treatments required concentrations between 1 and 5 ppm to effectively eliminate vegetative *A. catenella* cells (Fig. 3G-K). The oxidative biocides 4, 5-dichloro-N-octyl-4-isothiazolin-3-one, Hydrogen peroxide and Oxy-PYRION® 98% were effective at the respective concentrations of 60, 75 and 200 ppm following 48 h exposure (Fig 3L-N).

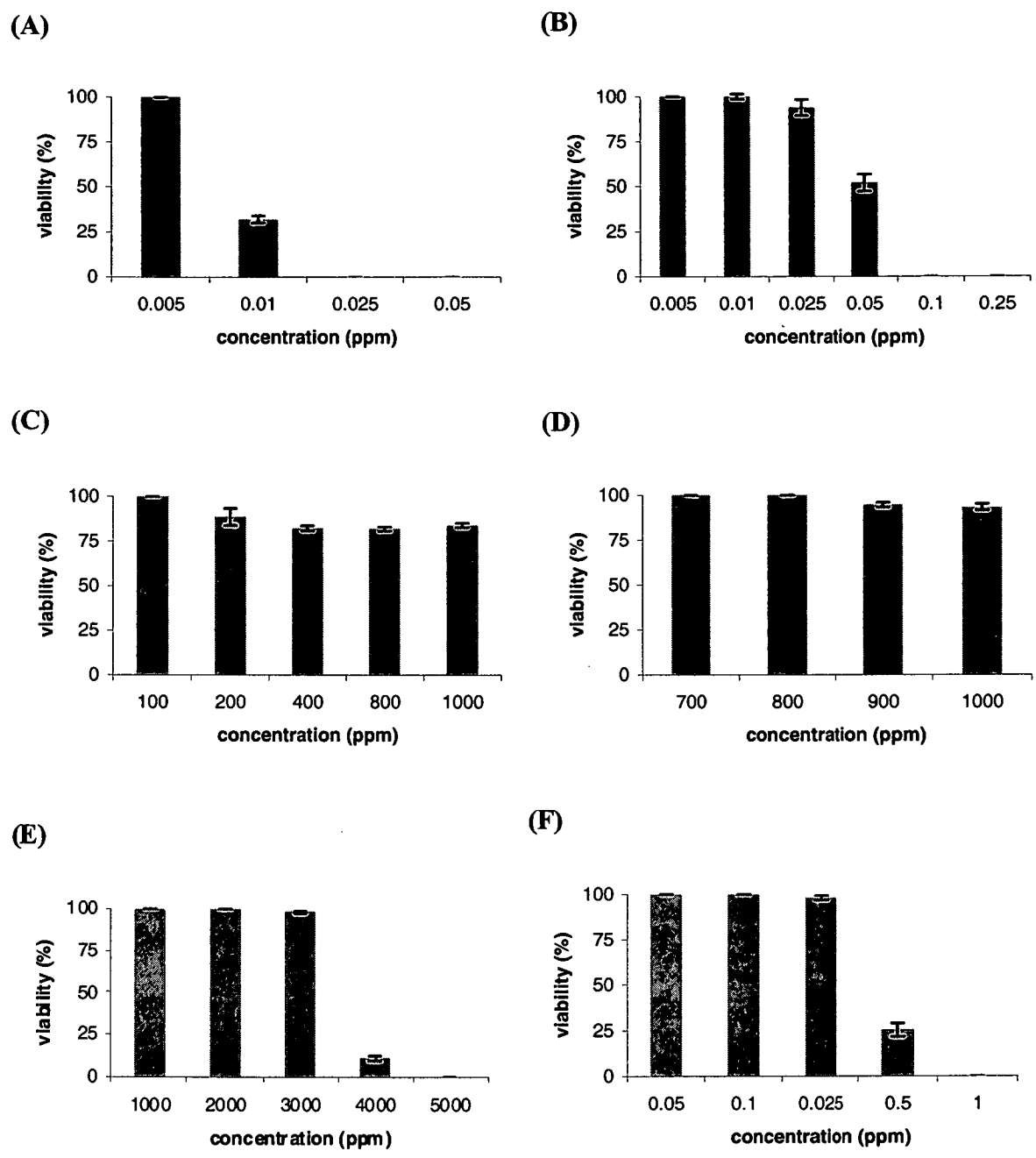


Fig. 3. Effect of varying concentrations of the chemical biocides on the viability of vegetative cells of the dinoflagellate *Alexandrium catenella* (48 h exposure). (A) Natrium PYRION® 40% treatment; (B) PYRION® Disulfide 40% Suspension treatment; C) Rimsulfuron PESTANAL® treatment; (D) Isoxaflutol PESTANAL® treatment; (E) LAg 2007 030 treatment; (F) KATHON™ 886F Biocide treatment. Bars indicate standard error.

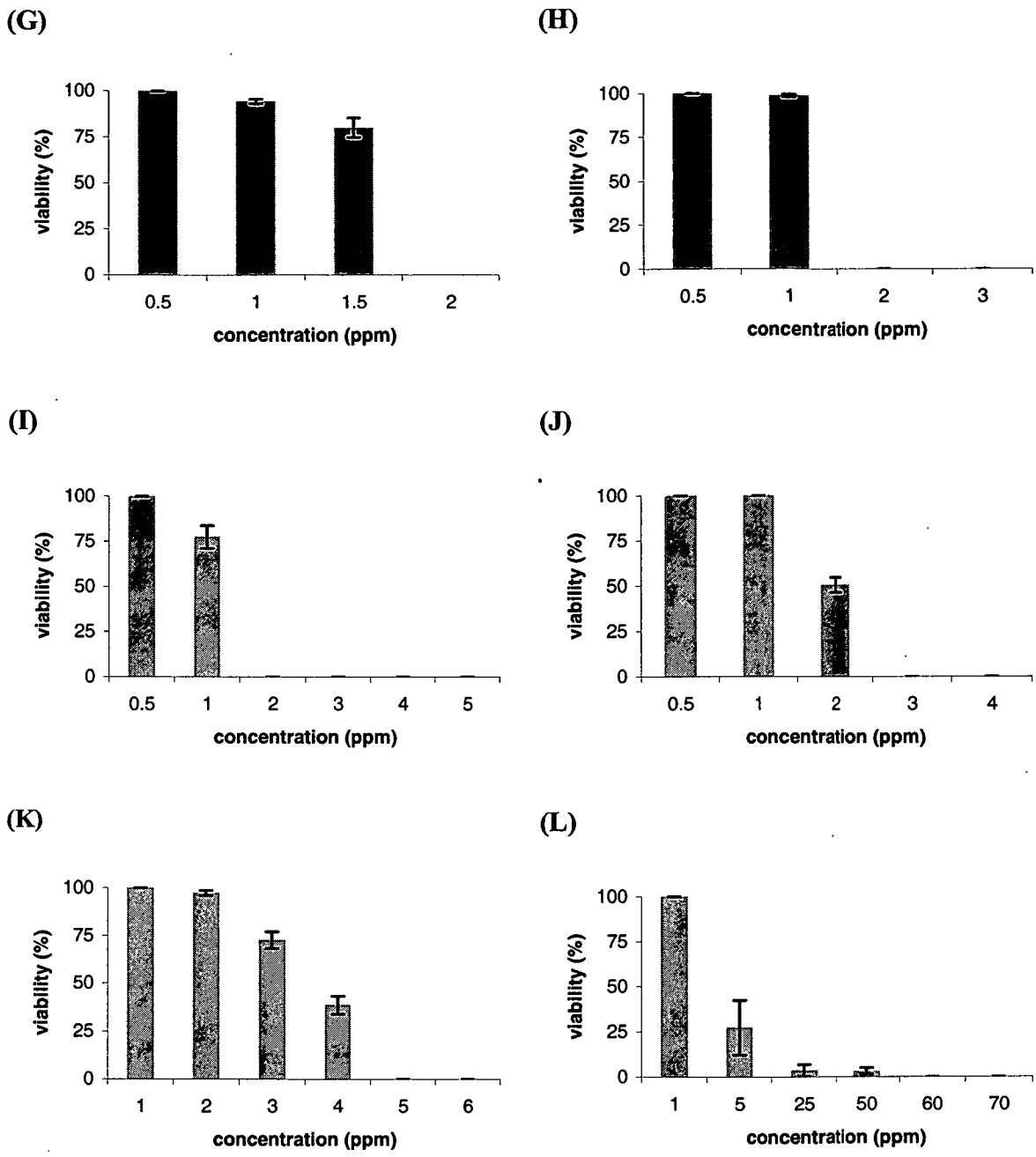
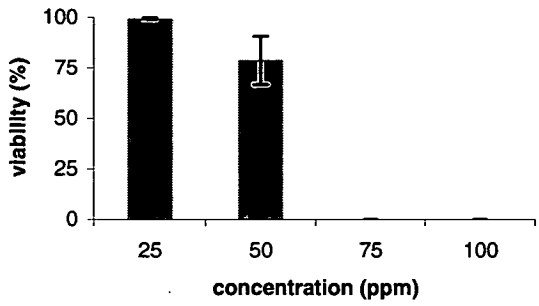


Fig. 3. *cont.*. Effect of varying concentrations of the chemical biocides on the viability of vegetative cells of the dinoflagellate *Alexandrium catenella* (48 h exposure). (G) ARQUAD MCB-50 treatment; (H) PROTECTNOL BN treatment. (I) R090026 treatment; (J) LAg 2006 041 treatment; (K) Intercide OIT treatment; (L) 4, 5-dichloro-N-octyl-4-isothiazolin-3-one treatment. Bars indicate standard error.

(M)



(N)

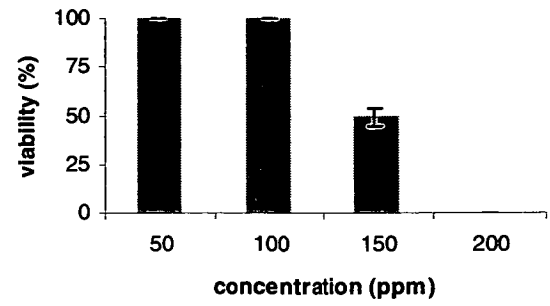


Fig. 3. *cont.*. Effect of varying concentrations of the chemical biocides on the viability of vegetative cells of the dinoflagellate *Alexandrium catenella* (48 h exposure). (M) Hydrogen peroxide 30% treatment; (N) Oxy-PYRION® 98% treatment. Bars indicate standard error.

The biocide concentrations required to eliminate vegetative cells of the haptophyte *Prymnesium parvum* and the green-flagellate *Tetraselmis suecica* are presented in Figures 4 and 5. Both these test species were found to be the most tolerant to ECONEA® Technical treatment. The majority of the chemical biocides eliminated vegetative cells of *P. parvum* and *T. suecica* at the same, or very similar, concentrations required to kill *A. catenella* cells (Fig. 4 and 5). The Natrium PYRION® 40% and PYRION® Disulfide 40% biocides were slightly less effective against *P. parvum* and *T. suecica* requiring respective concentrations of 0.1 ppm and 0.5 ppm for the complete elimination of viable vegetative cells of both species. Increased concentrations of the biocide Lag 2006 041(10 ppm for *P. parvum* and 20 ppm for *T. suecica*) were needed for effective control compared to the 3 ppm required to eliminate *A. catenella* cells, and an increase of 1 ppm was required to control *T. suecica* using KATHON™ 886F Biocide. PROTECTNOL BN was not effective against *T. suecica* vegetative cells (Fig. 5F) at the maximum concentration tested (20 ppm), yet the biocide could control *P. parvum* (Fig. 4F) and *A. catenella* cells (Fig. 3H) at 2 ppm. Following an exposure period of 48 h, 65% of *T. suecica* cells remained viable following treatment with PROTECTNOL BN at a concentration of 20 ppm (Fig. 5F). The half maximal effective concentrations (EC50) of ECONEA® Technical and the different co-biocides are provided in Table 3 to allow the intercomparison between the effectiveness of ECONEA® Technical and the different co-biocides against *A. catenella, P. parvum* and *T. suecica.*

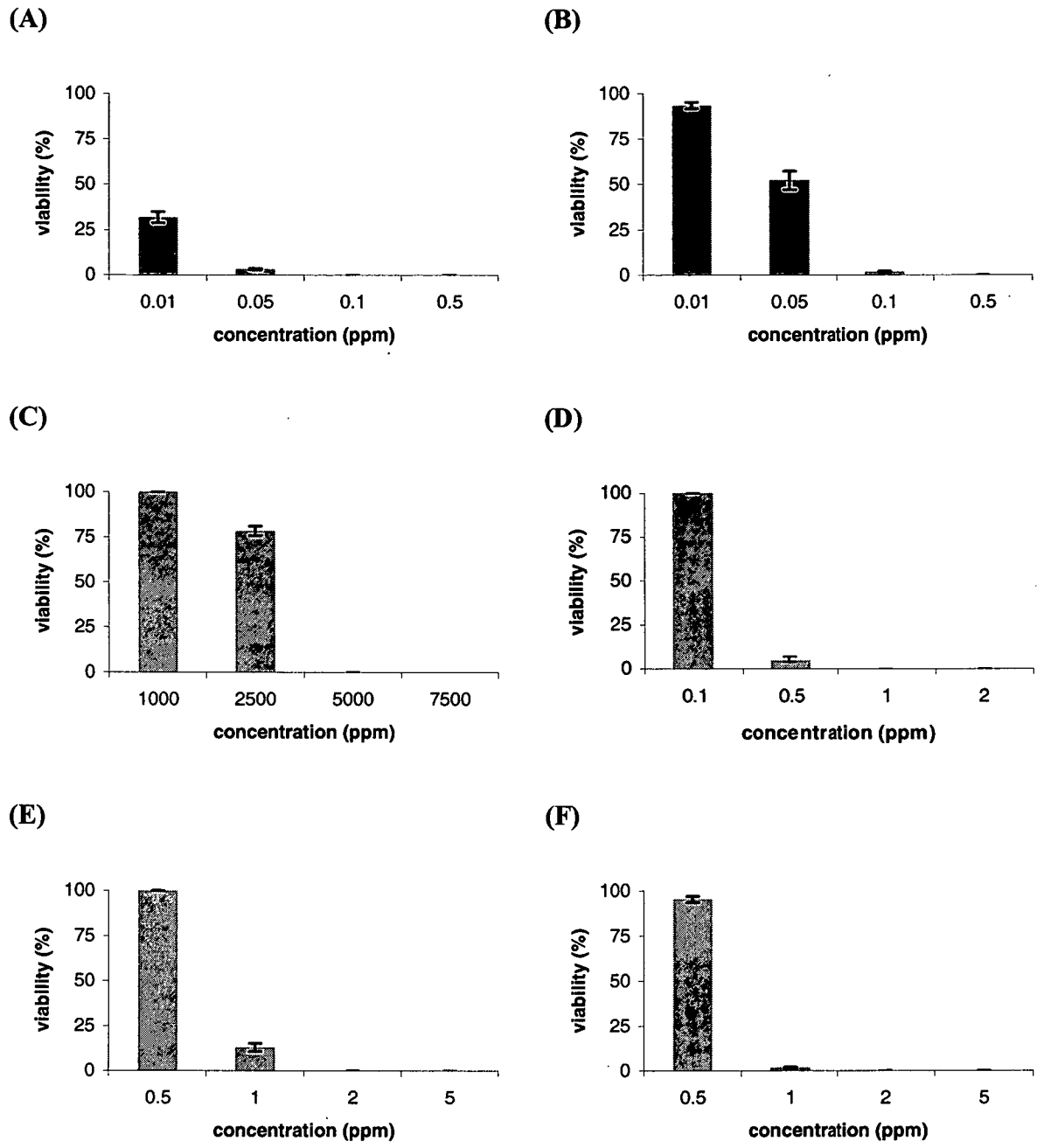


Fig. 4. Effect of varying concentrations of the chemical biocides on the viability of vegetative cells of the haptophyte *Prymnesium parvum* (48 h exposure). (A) Natrium PYRION® 40% treatment; (B) PYRION® Disulfide 40% Suspension treatment; (C) LAg 2007 030 treatment; (D) KATHON™ 886F Biocide treatment; (E) ARQUAD MCB-50 treatment; (F) PROTECTNOL BN treatment. Bars indicate standard error.

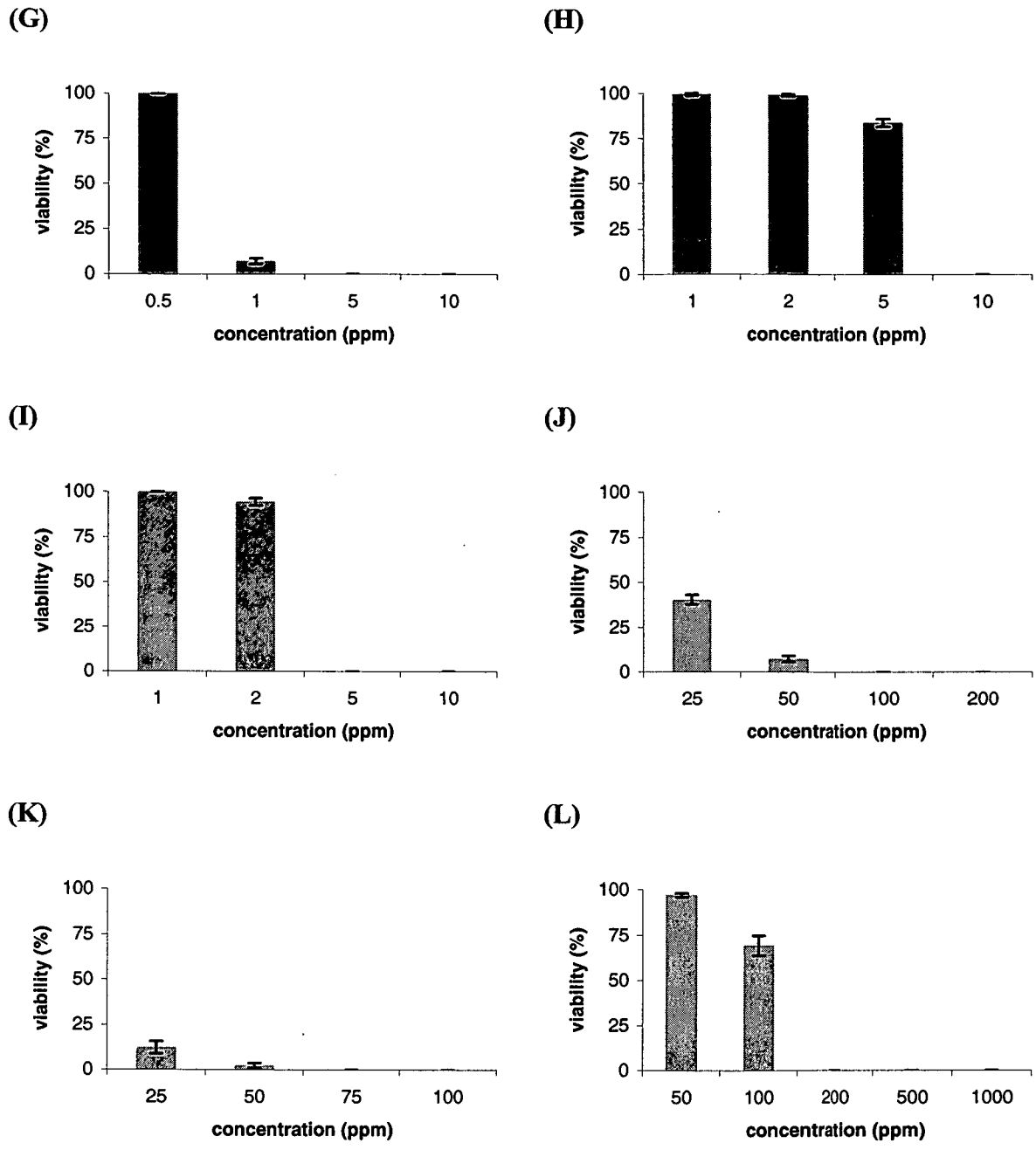


Fig. 4 *cont.*. Effect of varying concentrations of the chemical biocides on the viability of vegetative cells of the haptophyte *Prymnesium parvum* (48 h exposure). (G) R090026 treatment; (H) LAg 2006 041 treatment; (I) Intercide OIT treatment; (J) 4, 5-dichloro-N-octyl-4-isothiazolin-3-one treatment; (K) Hydrogen peroxide 30% treatment; (L) Oxy-PYRION® 98% treatment. Bars indicate standard error.

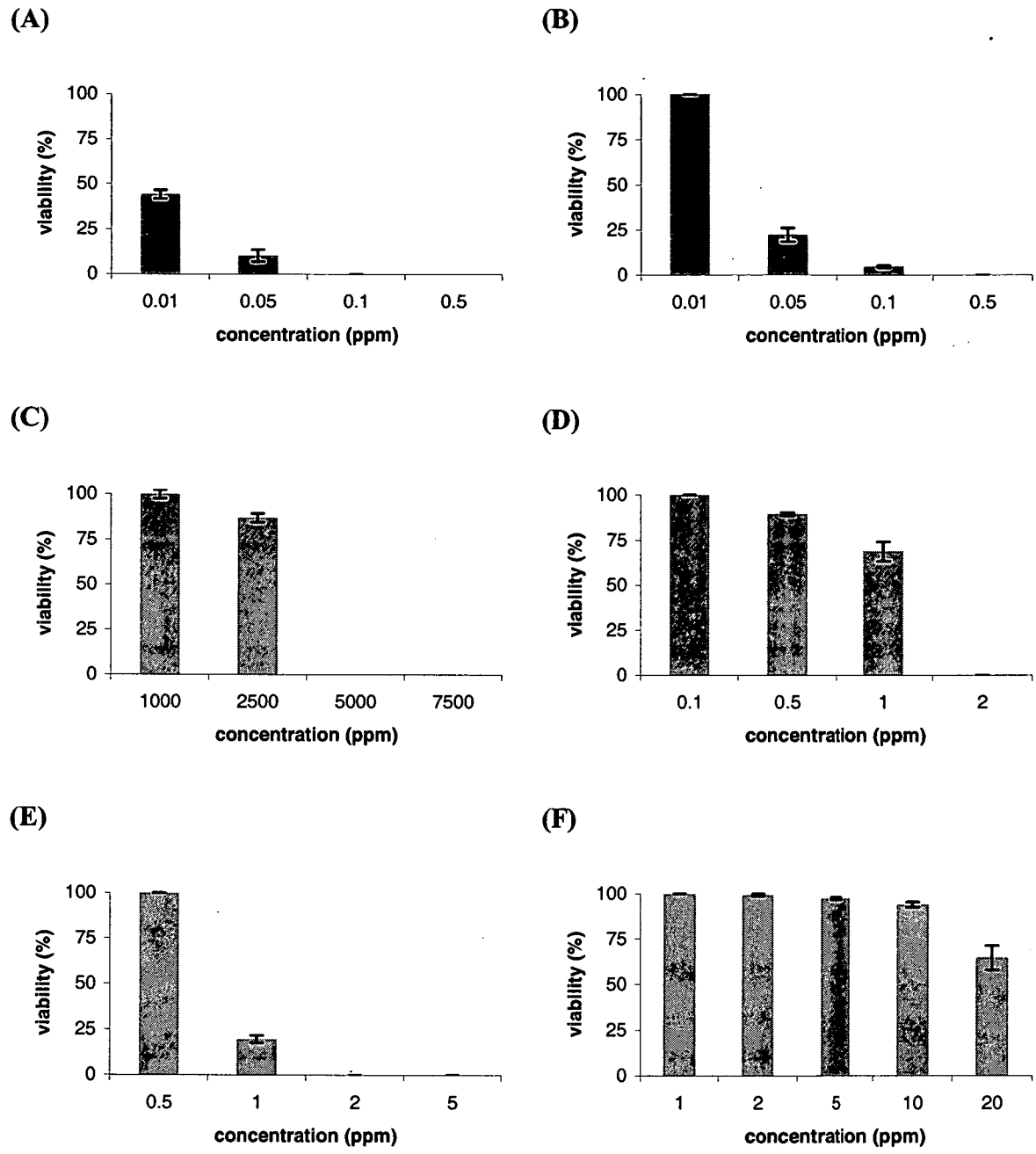


Fig. 5. Effect of varying concentrations of the chemical biocides on the viability of vegetative cells of the green-flagellate *Tetraselmis suecica* (48 h exposure). (A) Natrium PYRION® 40% treatment; (B) PYRION® Disulfide 40% Suspension treatment; (C) LAg 2007 030 treatment; (D) KATHON™ 886F Biocide treatment; (E) ARQUAD MCB-50 treatment; (F) PROTECTNOL BN treatment. Bars indicate standard error.

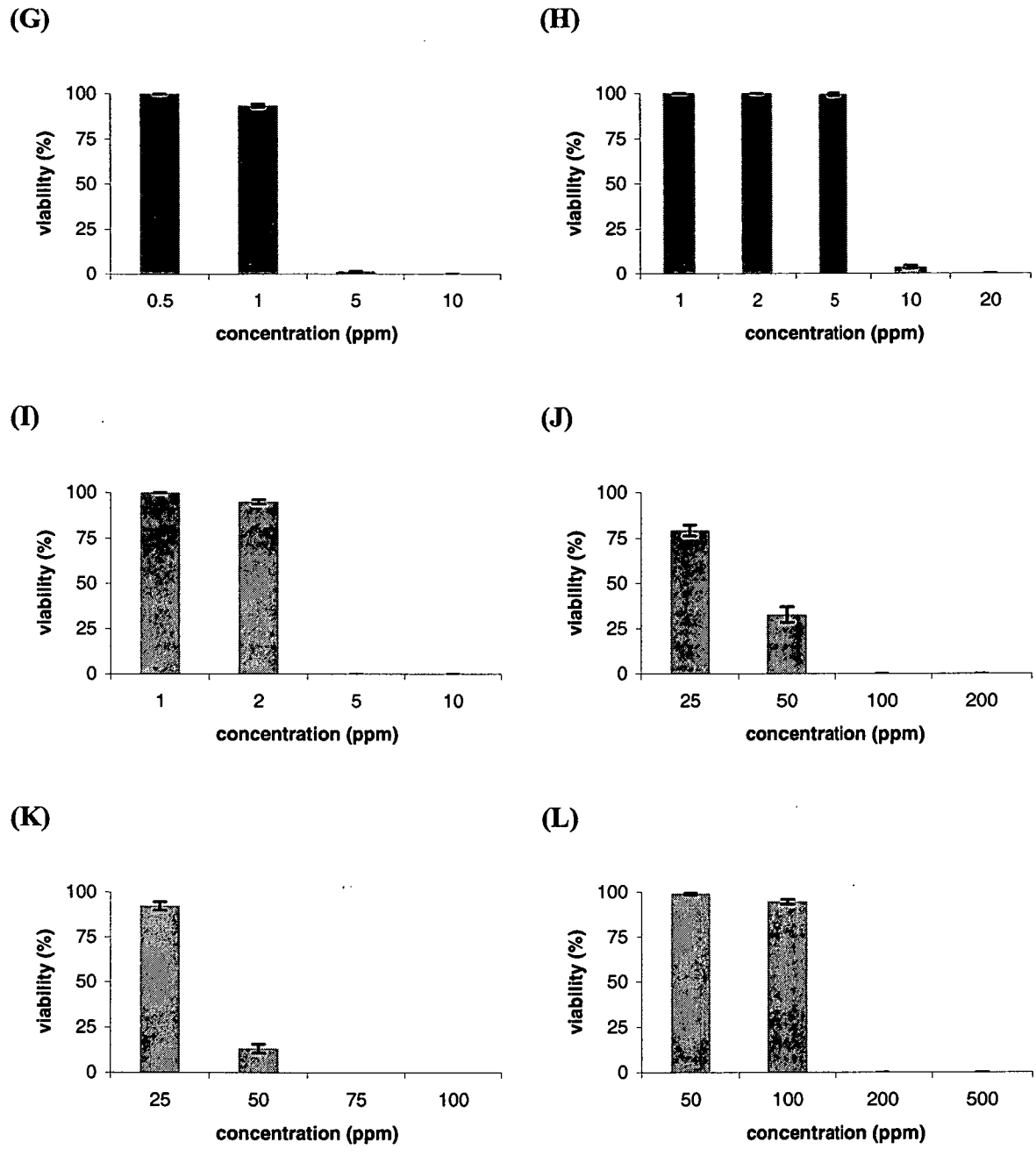


Fig. 5 *cont.*. Effect of varying concentrations of the chemical biocides on the viability of vegetative cells of the green-flagellate *Tetraselmis suecica* (48 h exposure). (G) R090026 treatment; (H) LAg 2006 041 treatment; (I) Intercide OIT treatment; (J) 4, 5-dichloro-N-octyl-4-isothiazolin-3-one treatment; (K) Hydrogen peroxide 30% treatment; (L) Oxy-PYRION® 98% treatment. Bars indicate standard error.

Table 3. Comparison of half maximal effective concentrations (EC50) of ECONEA® Technical and the 15 co-biocides against vegetative cells of *Alexandrium catenella*, *Karenia papilionacea* and *Tetraselmis suecica* (48 h exposure).

Product name	EC50 (ppm)		
	Alexandrium catenella	*Karenia papilionacea*	*Tetraselmis suecica*
ARQUAD MCB-50	1.5	0.85	0.95
ECONEA® Technical	0.08	0.75	NA
Hydrogen peroxide 30%	27	8	39
Intercide OIT	3.1	3.1	3.1
Isoxaflutol PESTANAL®	NA	NA	NA
KATHON™ 886F Biocide	0.24	0.28	0.92
LAg 2006 041	1.65	5.3	6.1
LAg 2007 030	3100	2800	2950
Natrium PYRION® 40%	0.005	0.005	0.005
Oxy-PYRION® 98%	135	140	143
PROTECTNOL BN	1.22	0.72	NA
PYRION® Disulfide 40% Suspension	0.04	0.04	0.04
Rimsulfuron PESTANAL®	NA	NA	NA
R090026	1.3	0.95	2.15
4, 5-dichloro-N-octyl-4-isothiazolin-3-one	5	13	40

Chemical treatment of dinoflagellate cysts

The biocide concentrations required for the inactivation of resistant sexual resting cysts of the dinoflagellate *Gymnodinium catenatum* are shown in Tables 4.1 to 4.15. For all treatments, considerably higher concentrations were required than those needed to kill vegetative cells. For ECONEA® Technical, complete inactivation was not achieved at the maximum concentration tested of 1000 ppm (Table 4.1) Following 2 weeks exposure to 1000 ppm ECONEA® Technical, the cell integrity of *G. catenatum* resting cysts was not compromised and internal cell contents appear undamaged (Fig. 6). Other biocides that performed poorly against *G. catenatum* cysts included LAg 2007 030, Isoxaflutol PESTANAL®, Rimsulfuron PESTANAL® (complete control not achieved at 10 000 ppm) and 4, 5-dichloro-N-octyl-4-isothiazolin-3-one (complete inactivation at 10 000 ppm). The lowest biocide concentrations required for the complete inactivation of *G. catenatum* cysts were 10 ppm (Natrium PYRION® 40% and PYRION® Disulfide 40% Suspension, Tables 3.6 and 3.7), and 25 ppm (PROTECTNOL BN, Table 4.8). ARQUAD MCB-50 and Intercide OIT were toxic at 100 ppm (Tables 4.9 and 4.10), whereas the remaining biocide required concentration of 500 ppm (KATHON™ 886F, Oxy-PYRION®

98%, LAg 2006 041), 1000 ppm (R090026) and 2500 ppm (Hydrogen peroxide 30%) to completely inactivate the dinoflagellate cysts (Tables 4.11 to 4.15).

Table 4.1. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of ECONEA® Technical.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	19/22	31/37	32/35	82/94	87.2 ± 1.4
0.1	16/22	39/44	32/39	87/105	82.9 ± 2.7
0.5	32/36	19/26	22/27	73/89	82 ± 2.6
2.5	27/32	21/34	33/44	81/110	73.6 ± 3.8
10	20/41	39/51	27/42	86/134	64.2 ± 4.6
50	23/44	27/43	72/109	122/196	62.2 ± 2.4
250	19/67	50/109	32/55	101/231	43.7 ± 5
1000	22/59	47/99	16/52	85/210	40.5 ± 2.8

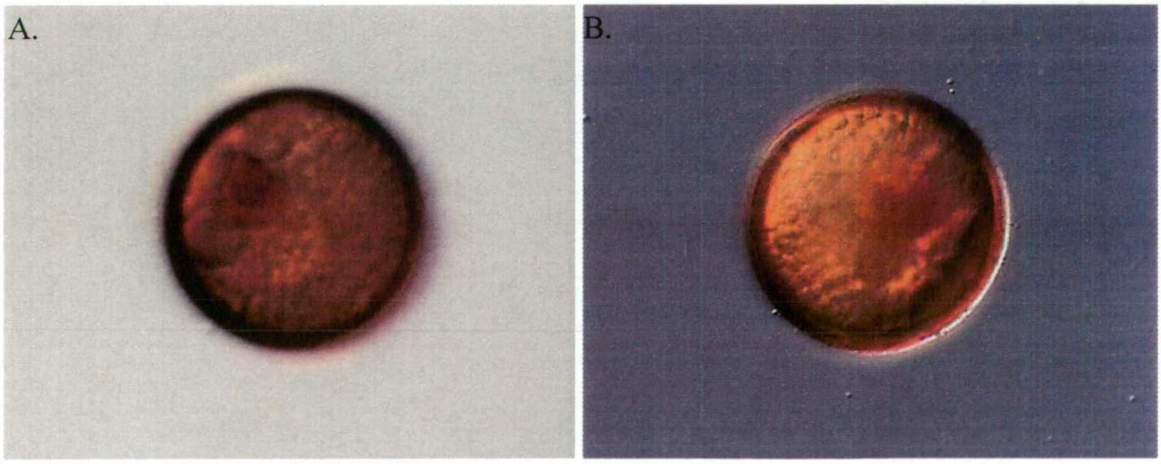


Fig. 6. *Gymnodinium catenatum* resting cyst exposed to ECONEA® Technical. (A) Control treatment; (B) two weeks exposure to 1000 ppm.

Table 4.2. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of LAg 2007 030.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	31/33	39/42	22/27	92/102	90.2 ± 2.3
100	40/52	44/52	32/37	116/141	82.3 ± 1.7
500	36/42	30/40	53/60	119/142	83.8 ± 2.5
2500	32/50	41/49	51/58	124/157	79 ± 4.3
10000	29/39	22/29	39/47	90/115	78.3 ± 1.5

Table 4.3. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of Isoxaflutol PESTANAL®.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	20/20	29/32	28/31	77/83	92.8 ± 1.8
100	28/29	38/42	30/35	96/106	90.6 ± 1.8
500	19/21	31/34	36/43	86/98	87.8 ± 1.4
2500	22/28	25/38	21/25	68/91	74.7 ± 3.1
10000	9/16	28/36	37/45	74/97	76.3 ± 4.6

Table 4.4. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of Rimsulfuron PESTANAL®.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	29/36	31/41	35/37	95/114	83.3 ± 3.3
100	35/50	27/42	31/48	93/140	66.4 ± 1.1
500	19/39	21/48	12/41	52/128	40.6 ± 3.4
2500	12/29	24/37	21/39	57/105	54.3 ± 3.9
10000	12/31	12/22	28/41	52/94	55.3 ± 4.9

Table 4.5. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of 4, 5-dichloro-N-octyl-4-isothiazolin-3-one.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	29/32	39/44	41/45	109/121	90.1 ± 0.4
100	71/86	38/46	29/39	138/171	80.7 ± 1.6
500	62/79	39/52	40/59	141/190	74.2 ± 1.8
2500	39/60	22/51	19/50	80/161	49.7 ± 4.8
10000	0/29	0/52	0/44	0/125	0

Table 4.6. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of Natrium PYRION® 40%.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	37/42	26/28	29/42	92/112	82.1 ± 4.2
0.1	28/34	20/27	22/27	70/88	79.5 ± 1.5
0.5	14/27	22/40	13/25	49/92	53.3 ± 0.6
2.5	16/30	12/22	12/29	40/81	49.4 ± 2.4
10	0/16	0/18	0/40	0/74	0
50	0/62	0/41	0/44	0/147	0

Table 4.7. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of PYRION® Disulfide 40% Suspension.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	22/24	32/37	16/23	70/84	83.3 ± 3.9
0.1	36/43	29/33	27/35	92/111	82.9 ± 1.8
0.5	17/37	19/29	22/42	58/108	53.7 ± 3.3
2.5	33/49	23/40	19/32	75/121	62 ± 1.7
10	0/28	0/32	0/29	0/89	0

Table 4.8. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of PROTECTNOL BN.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	32/37	30/40	41/47	103/124	83.1 ± 2.3
1	49/55	51/57	42/56	142/168	84.5 ± 2.8
5	19/50	16/42	12/40	47/132	35.6 ± 1.5
25	0/60	0/39	0/47	0/146	0
100	0/52	0/51	0/38	0/141	0

Table 4.9. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of ARQUAD MCB-50.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	51/54	32/36	40/43	123/133	92.5 ± 1
1	30/39	22/28	13/41	65/108	60.2 ± 8.9
2.5	29/38	21/31	36/52	86/121	71.1 ± 1.5
25	7/51	13/59	6/32	26/142	18.3 ± 1.4
100	0/44	0/60	0/29	0/133	0

Table 4.10. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of Intercide OIT.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	31/33	42/49	22/27	95/109	87.2 ± 2.1
5	12/19	22/39	20/40	54/98	55.1 ± 2.2
25	20/27	9/19	12/22	41/68	60.3 ± 4.6
100	0/37	0/41	0/45	0/123	0
500	0/21	0/37	0/42	0/100	0

Table 4.11. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of KATHON™ 886F Biocide.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	31/38	47/51	39/45	117/134	87.3 ± 1.8
1	49/51	39/42	29/31	117/124	94.4 ± 0.6
5	30/40	32/46	29/41	91/127	71.7 ± 1
25	21/41	37/45	22/48	80/134	59.7 ± 6.5
100	4/58	0/37	4/49	8/144	5.6 ± 1.5
500	0/71	0/50	0/31	0/152	0

Table 4.12. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of Oxy-PYRION® 98%.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	51/60	37/41	32/42	120/143	83.9 ± 2.4
100	30/40	29/43	37/50	96/133	72.2 ± 1.4
500	0/77	0/41	0/35	0/153	0
2500	0/40	0/39	0/71	0/150	0
10000	0/42	0/60	0/57	0/159	0

Table 4.13. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of LAg 2006 041.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	32/42	26/31	19/26	77/99	77.8 ± 1.9
5	31/58	22/40	24/39	77/137	56.2 ± 1.4
25	9/28	19/40	13/46	41/114	36 ± 3.7
100	4/41	3/49	5/42	12/132	9.1 ± 1
500	0/30	0/19	0/27	0/76	0

Table 4.14. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of R090026.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	41/47	52/55	33/38	126/140	90 ± 1.4
1	27/30	22/26	27/32	76/88	86.4 ± 1.1
5	33/40	37/41	12/19	82/100	82 ± 4.6
25	18/26	22/31	17/22	57/79	72.2 ± 1.4
100	6/37	7/25	12/39	25/101	24.8 ± 2.6
500	3/51	4/58	2/45	9/154	5.8 ± 0.4
1000	0/61	0/92	0/47	0/200	0

Table 4.15. Germination success of resting cysts of the dinoflagellate *G. catenatum* after 2 weeks exposure to different concentrations of Hydrogen peroxide 30%.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	41/49	42/44	37/41	120/134	89.6 ± 2
100	59/62	50/54	29/33	138/149	92.6 ± 1.2
500	31/42	29/41	33/40	93/123	75.6 ± 2
1000	9/44	7/51	9/34	25/129	19.4 ± 2.1
2500	0/27	0/41	0/67	0/135	0

ECONEA™ *degradation*

The degradation of 1 ppm of ECONEA® Technical is displayed in Figure 7. At a concentration of 1 ppm, all ECONEA® Technical samples were found to degrade to a level non-toxic to all four species of microalgae (*G. catenatum, A. catenella, P. reticulatum, C. marina*) within 4-6 weeks under conditions of 12 h light/12 h dark at 17°C (Fig. 7). ECONEA® Technical degraded at different rates depending on light conditions, sediment load and water type. All samples degraded considerably slower when kept in the dark, requiring 8-12 weeks before samples were non-toxic to all four microalgal species. The presence of sediment increased the degradability of ECONEA® Technical. Under conditions of 12 h light/12 h dark, a 1 ppm concentration prepared in filtered seawater degraded to a non-toxic level in 6 weeks (Fig. 7A), compared to 4 weeks for samples prepared in filtered seawater containing

0.1, 0.5 and 1 g of ballast tank sediment (Fig. 7B-D). When the biocide was prepared in natural estuarine water from the Derwent River, samples degraded in 4 weeks (Fig. 7E), comparable to the degradation rates of the sediment trials, whilst the samples prepared in water collected from the humus-rich Huon River degraded noticeably slower, requiring 6 weeks to become non-toxic (Fig. 7F). When stored in the dark, the 0.5 and 1 g sediment samples degraded the fastest (8 weeks) (Fig. 7B, C), whereas the samples prepared in filtered seawater and Huon River estuarine water degraded the slowest, requiring 10 and 12 weeks respectively (Fig. 7A, F).

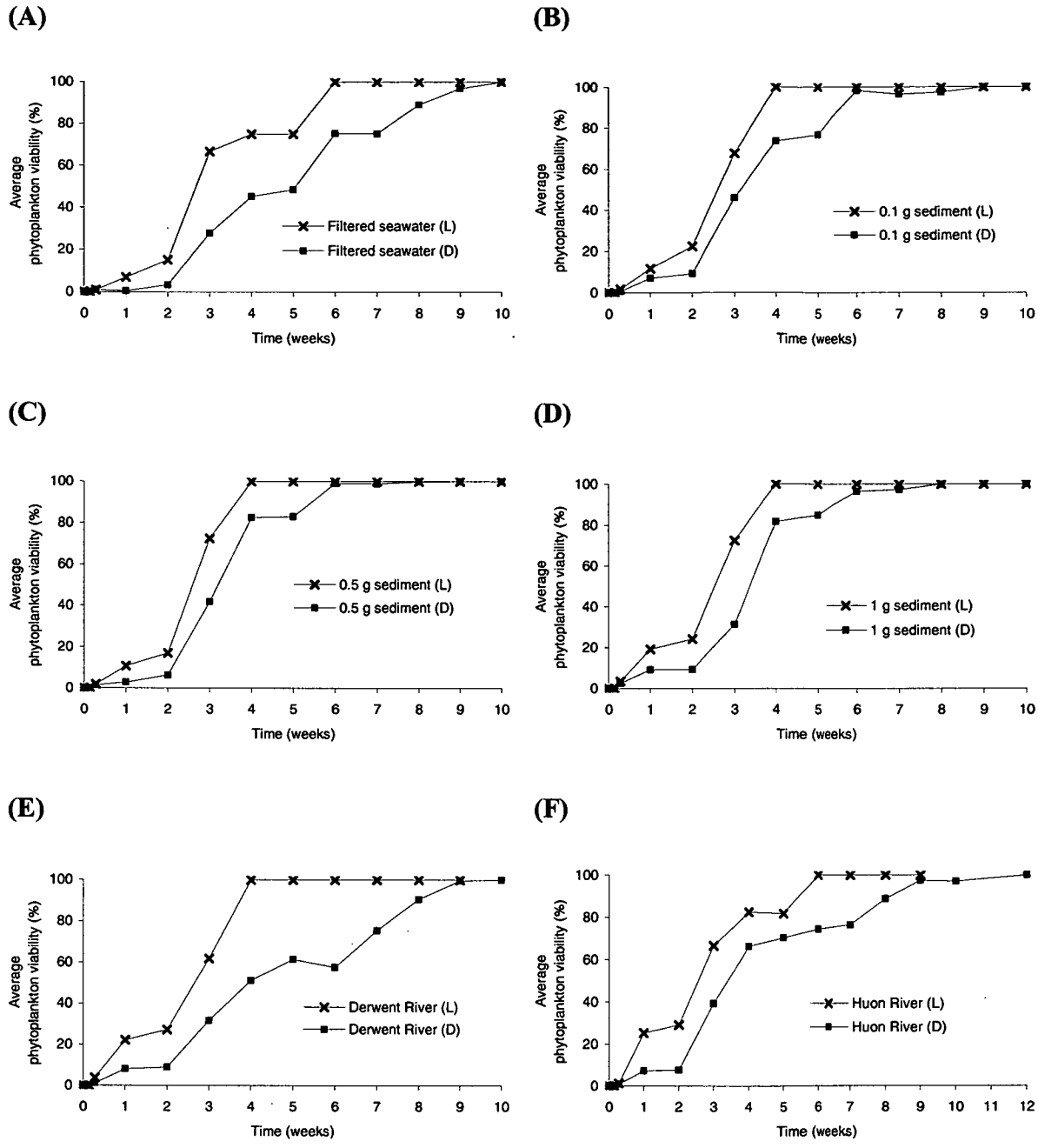


Fig. 7. Degradation of 1 ppm ECONEA® Technical (indirect estimate). Degradation assessed weekly by applying the ageing 1 ppm biocide concentrations to four separate vegetative microalgal species (*A. catenella, G. catenatum, P. reticulatum, C. marina*). (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

Figures 8 to 11 give a comparison of the differing sensitivity of the four microalgal test species (*A. catenella, G. catenatum, P. reticulatum, C. marina*) to the ageing 1 ppm ECONEA® Technical samples. The toxicity of the ageing 1 ppm samples varied considerably between the four test microalgal species. *Alexandrium catenella* and *Protoceratium reticulatum* were the least sensitive. When prepared in filtered seawater, 1 ppm concentration degraded to a level non-toxic to *A. catenella* vegetative cells in 4 weeks under conditions of 12 h light/12 h dark (Fig. 8A). Under the same light conditions, the time period required for the biocide to become non-toxic to *A. catenella* was reduced to 3 weeks when biocide solution were prepared in natural estuarine water and filtered seawater containing 0.1, 0.5 and 1 g of ballast sediment (Fig. 8B-D). The time taken for the 1 ppm ECONEA® Technical samples to become non-toxic to *A. catenella* was increased by 1 week when stored in the dark and was not influenced by the sediment load and water conditions. When exposed to *P. reticulatum* cells, complete degradation of the 1 ppm ECONEA® Technical samples occurred in 4 weeks when stored under 12 h light/ 12 h dark (Fig. 10). Under dark conditions, complete degradation occurred after 5 weeks for the sediment samples (Fig. 10B-D), and 6 weeks for the samples prepared in filtered seawater and natural estuarine water (Fig. 10A, E, F). This reduction in degradability of dark-stored ECONEA® Technical was most pronounced in the *G. catenatum* and *C. marina* trials. Dark-stored samples took 2-4 weeks longer to become non-toxic to *G. catenatum* (Fig. 9), and an extra 5-7 weeks were required for the samples to not impact on *C. marina* viability (Fig. 11).

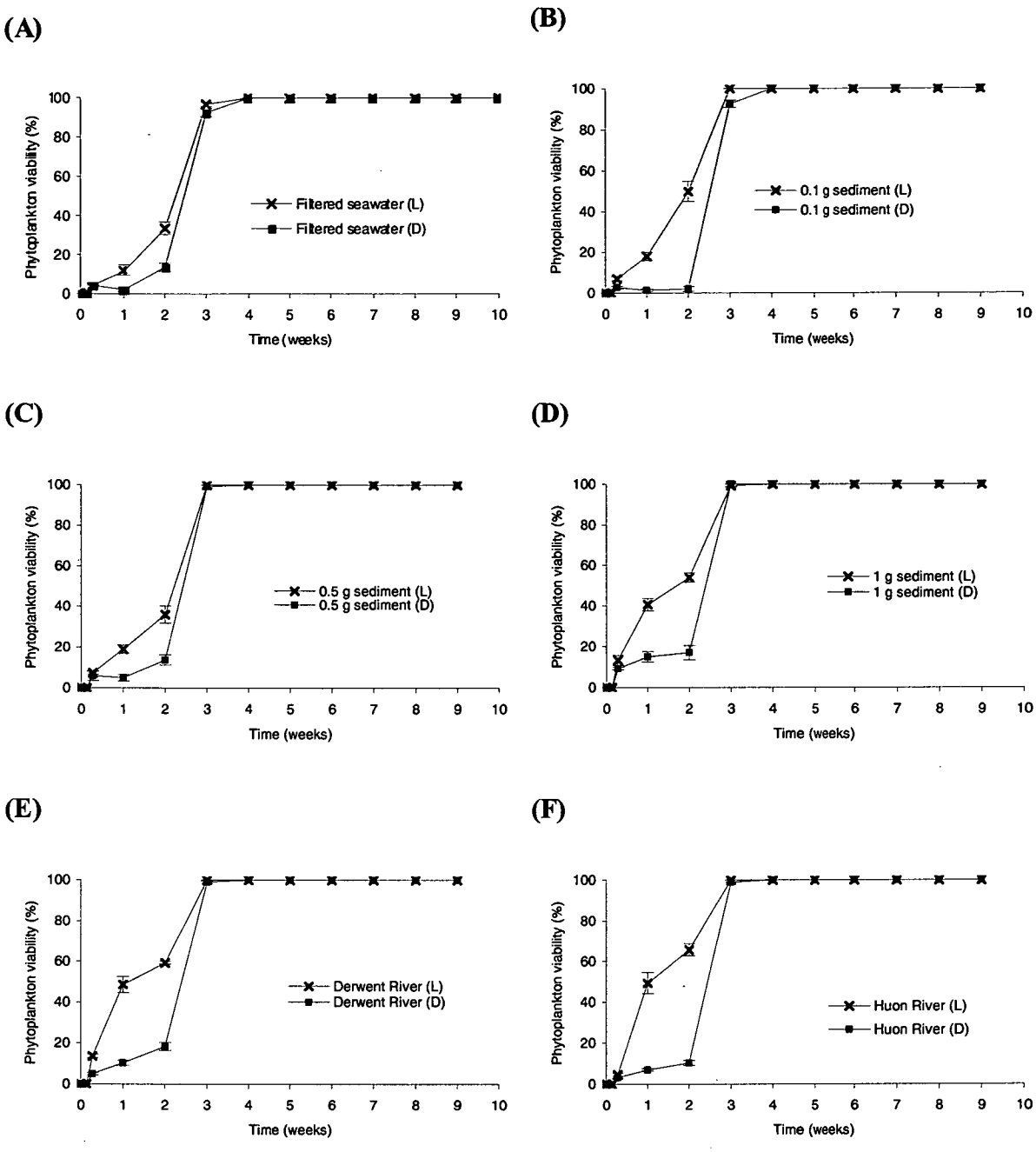


Fig. 8. Sensitivity of *Alexandrium catenella* to the ageing 1 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 1 ppm biocide concentrations to vegetative cells of the dinoflagellate *Alexandrium catenella*. (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

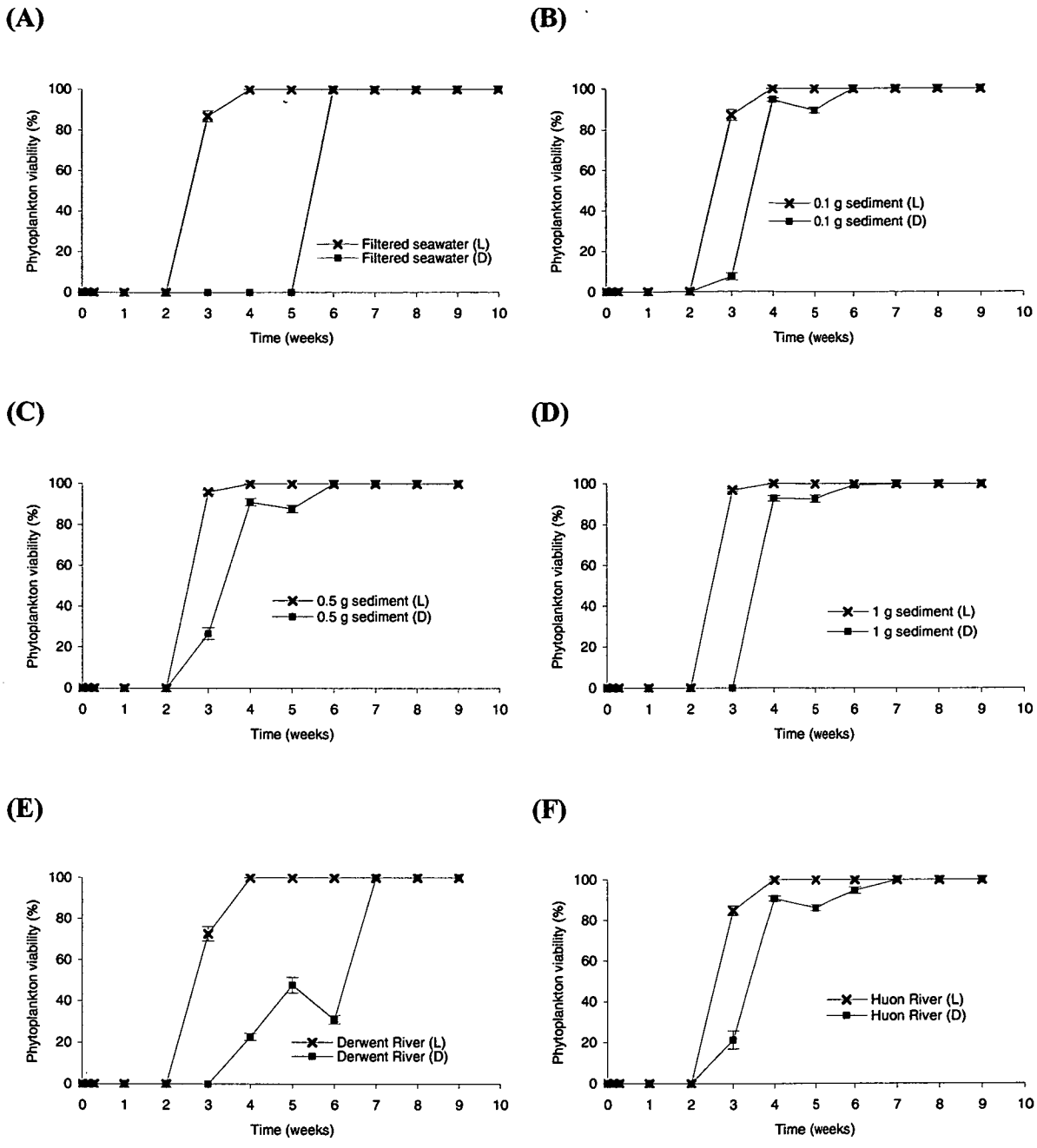


Fig. 9. Sensitivity of *Gymnodinium catenatum* to the ageing 1 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 1 ppm biocide concentrations to vegetative cells of the dinoflagellate *Gymnodinium catenatum.* (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

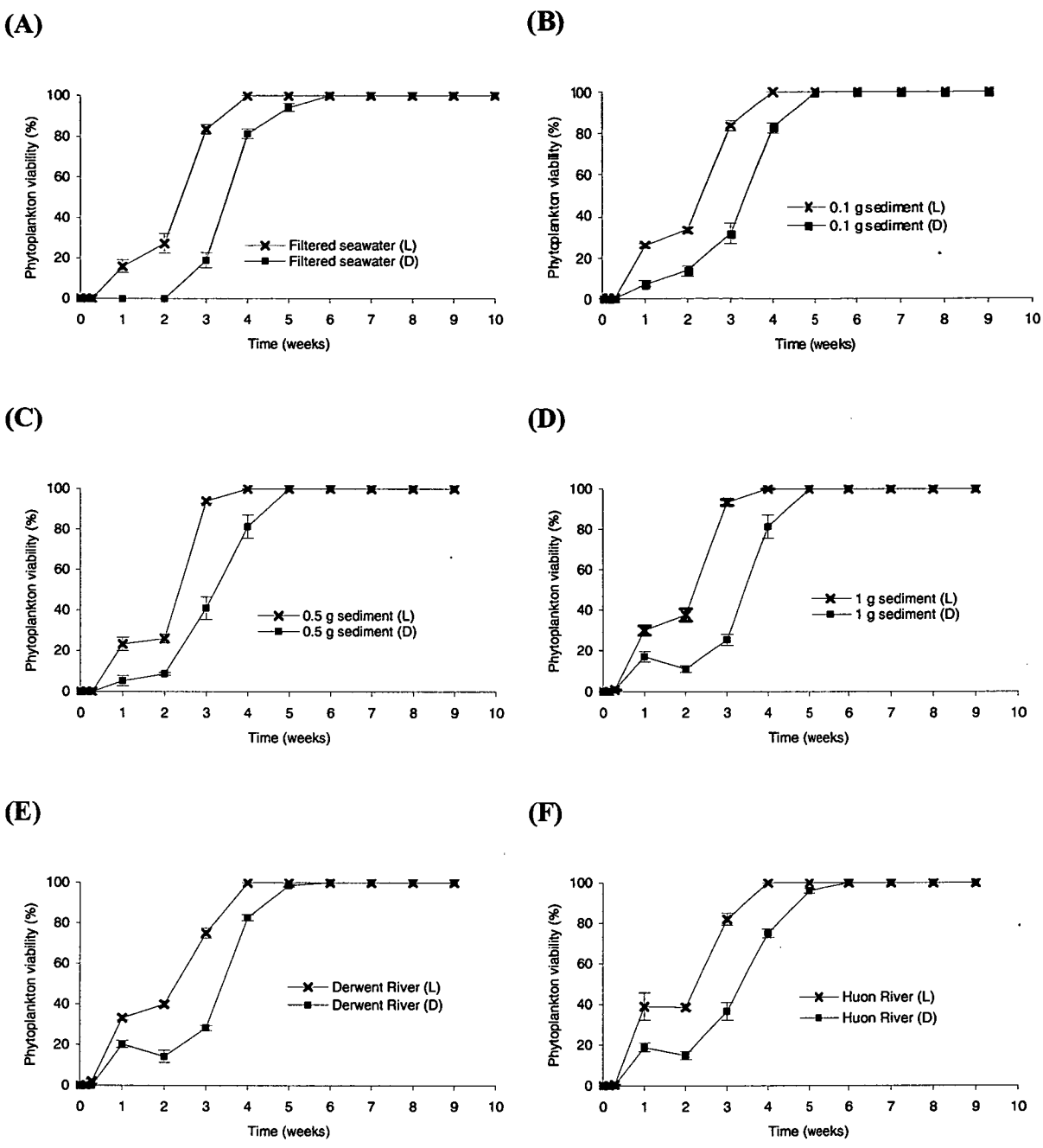


Fig. 10. Sensitivity of *Protoceratium reticulatum* to the ageing 1 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 1 ppm biocide concentrations to vegetative cells of the dinoflagellate *Protoceratium reticulatum.* (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

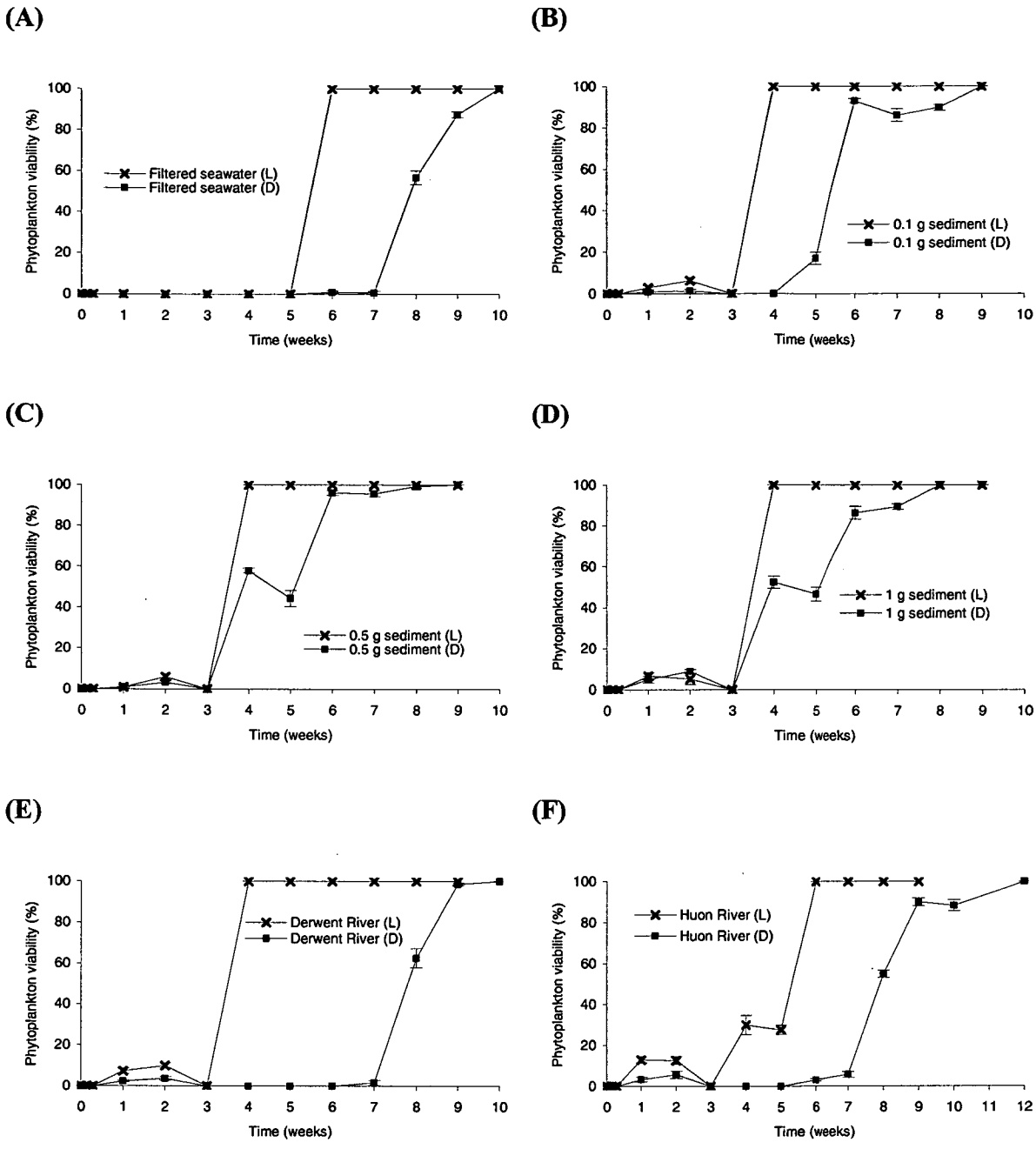


Fig. 11. Sensitivity of *Chattonella marina* to the ageing 1 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 1 ppm biocide concentrations to vegetative cells of the raphidophyte *Chattonella marina*. (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

Figure 12 displays the degradation of the 10 ppm ECONEA® Technical samples. The influence of sediment load, water type and light conditions was much more pronounced in the 10 ppm trials compared to the 1 ppm experiments. The biocide degraded considerably faster when exposed to light compared to dark-stored samples, and the presence of sediment reduced biocide toxicity when stored under 12 h light/ 12 h dark (Fig. 12). One hundred percent viability of all microalgal species was only achieved in the sediment samples stored under 12 h light/ 12 h dark (Fig. 12B-D). All other biocide samples still impacted on the phytoplankton cell viability of one or more of the test species following a period of 16 weeks. The biocide remained the most toxic when prepared in filtered seawater and the natural estuarine waters of the Huon and Derwent Rivers (Fig. 12A, E, F)

Alexandrium catenella and *P. reticulatum* were again identified as the least sensitive to the ageing 10 ppm biocide samples. When prepared in filtered seawater, a 10 ppm ECONEA® Technical concentration degraded to a level non-toxic to *A. catenella* in 8 weeks when exposed to 12 h light/12 h dark (Fig. 13A). Under the same conditions, 9 and 14 weeks were required to achieve 100% cell viability when tested against *P. reticulatum* (Fig. 15A) and *G. catenatum* (Fig. 14A) respectively. The ageing 10 ppm ECONEA® Technical samples prepared in filtered seawater were most toxic to *C. marina*. When exposed to light, samples did not degrade to a non-toxic level after 16 weeks (Fig. 16). The presence of ballast tank sediment resulted in a decrease in toxicity of ECONEA® Technical against all four microalgal test species. For example, the biocide degraded to a non-toxic level 2 weeks faster in the *A. catenella* sediment trials (Fig. 13B-D) and did not impact on *C. marina* cell viability after 10-12 weeks when samples were kept under 12 h light/12 h dark (Fig. 16B-D). All dark stored samples degraded considerably slower in all the microalgal trials. The additional time required for the dark-stored samples to become non-toxic when compared to the samples kept in 12 h light/ 12 h dark varied from 0 to 5 weeks for the *A. catenella* trials (Fig. 13), 4 to >16 weeks for the *P. reticulatum* trials (Fig. 15), and 6 to >16 weeks for the *G. catenatum* trials (Fig. 14). The biocide failed to degrade to a non-toxic level against *C. marina* when samples were stored in the dark (Fig. 16).

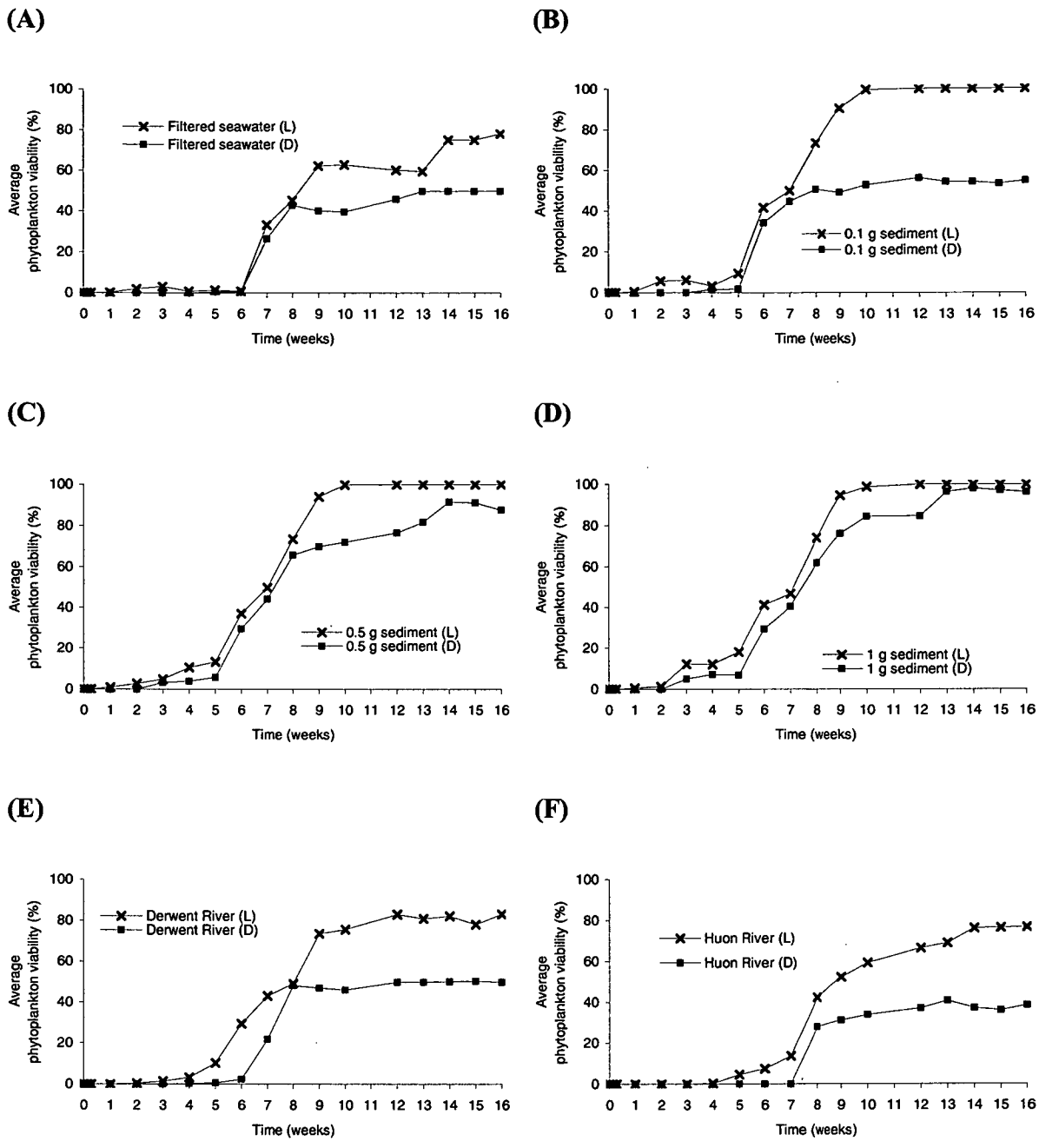


Fig. 12. Degradation of 10 ppm ECONEA® Technical (indirect estimate). Degradation assessed weekly by applying the ageing 10 ppm biocide concentrations to four separate vegetative microalgal species (*A. catenella, G. catenatum, P. reticulatum, C. marina*). (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. All samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

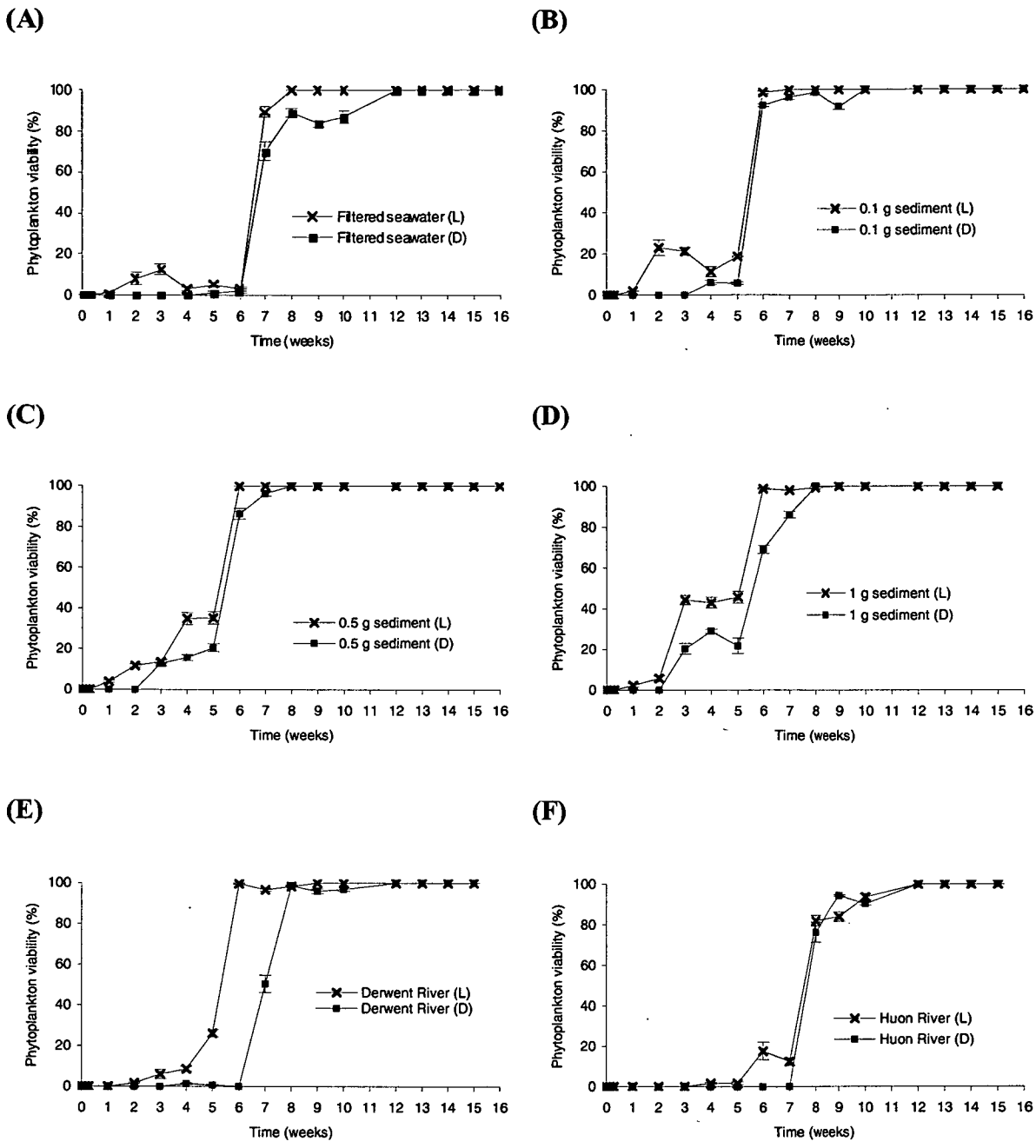


Fig. 13. Sensitivity of *Alexandrium catenella* to the ageing 10 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 10 ppm biocide concentrations to vegetative cells of the dinoflagellate *Alexandrium catenella.* **(A)** in filtered seawater. **(B)** in filtered seawater containing 0.1 g of ballast sediment. **(C)** in filtered seawater containing 0.5 g of ballast sediment. **(D)** in filtered seawater containing 1 g of ballast sediment. **(E)** in seawater collected from the Derwent River. **(F)** in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

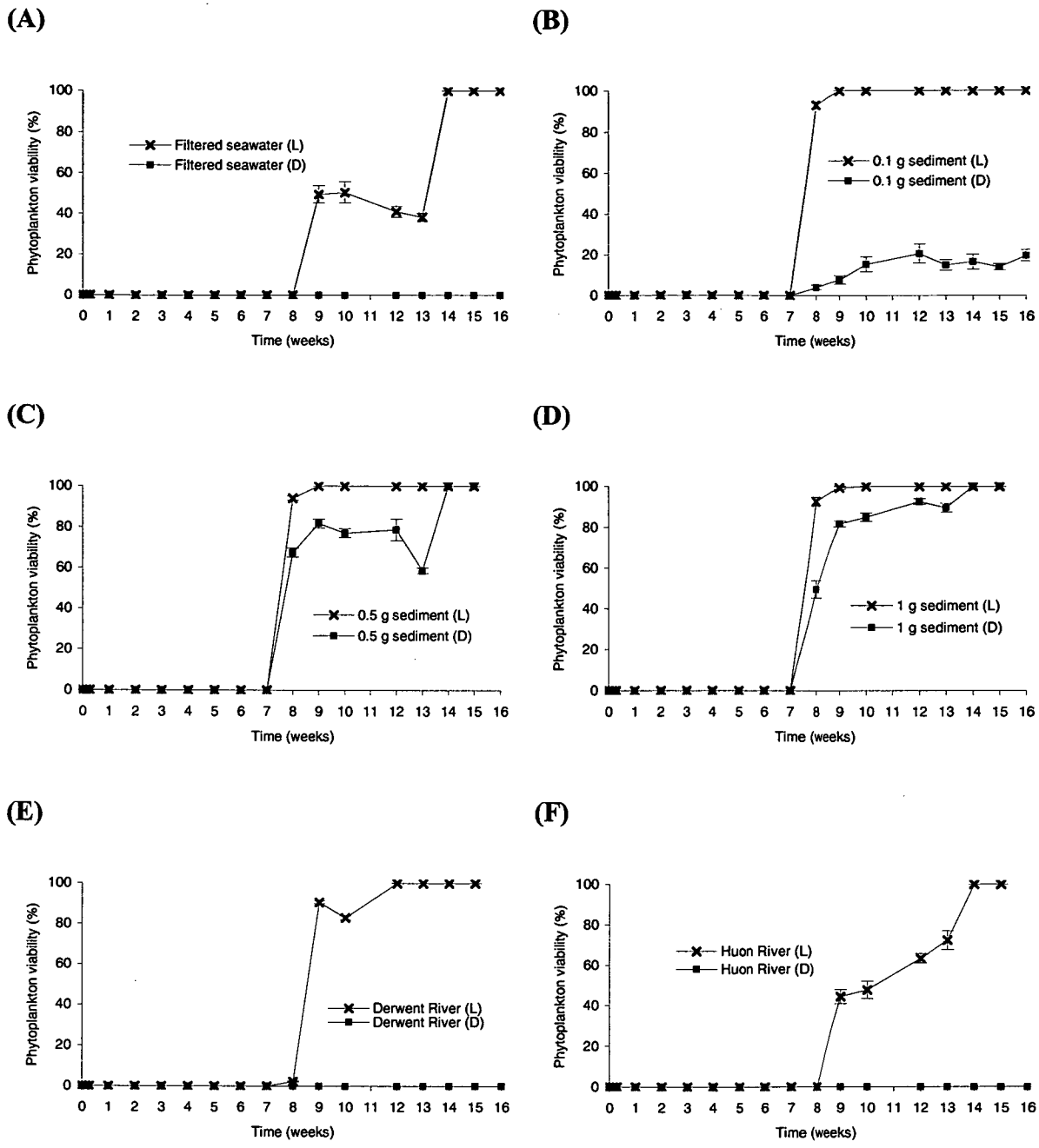


Fig. 14. Sensitivity of *Gymnodinium catenatum* to the ageing 10 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 10 ppm biocide concentrations to vegetative cells of the dinoflagellate *Gymnodinium catenatum.* (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

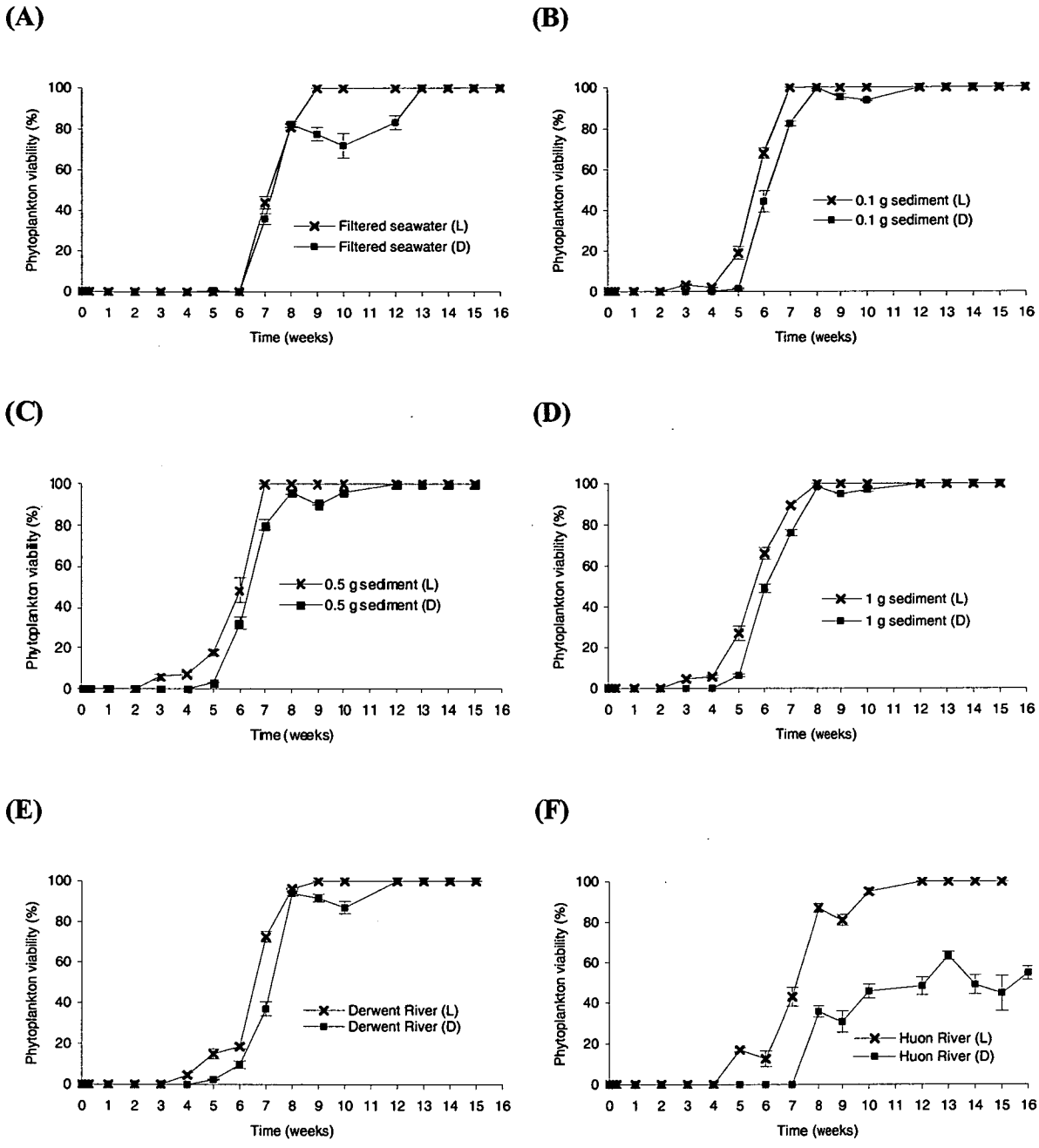


Fig. 15. Sensitivity of *Protoceratium reticulatum* to the ageing 10 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 10 ppm biocide concentrations to vegetative cells of the dinoflagellate *Protoceratium reticulatum.* **(A)** in filtered seawater. **(B)** in filtered seawater containing 0.1 g of ballast sediment. **(C)** in filtered seawater containing 0.5 g of ballast sediment. **(D)** in filtered seawater containing 1 g of ballast sediment. **(E)** in seawater collected from the Derwent River. **(F)** in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

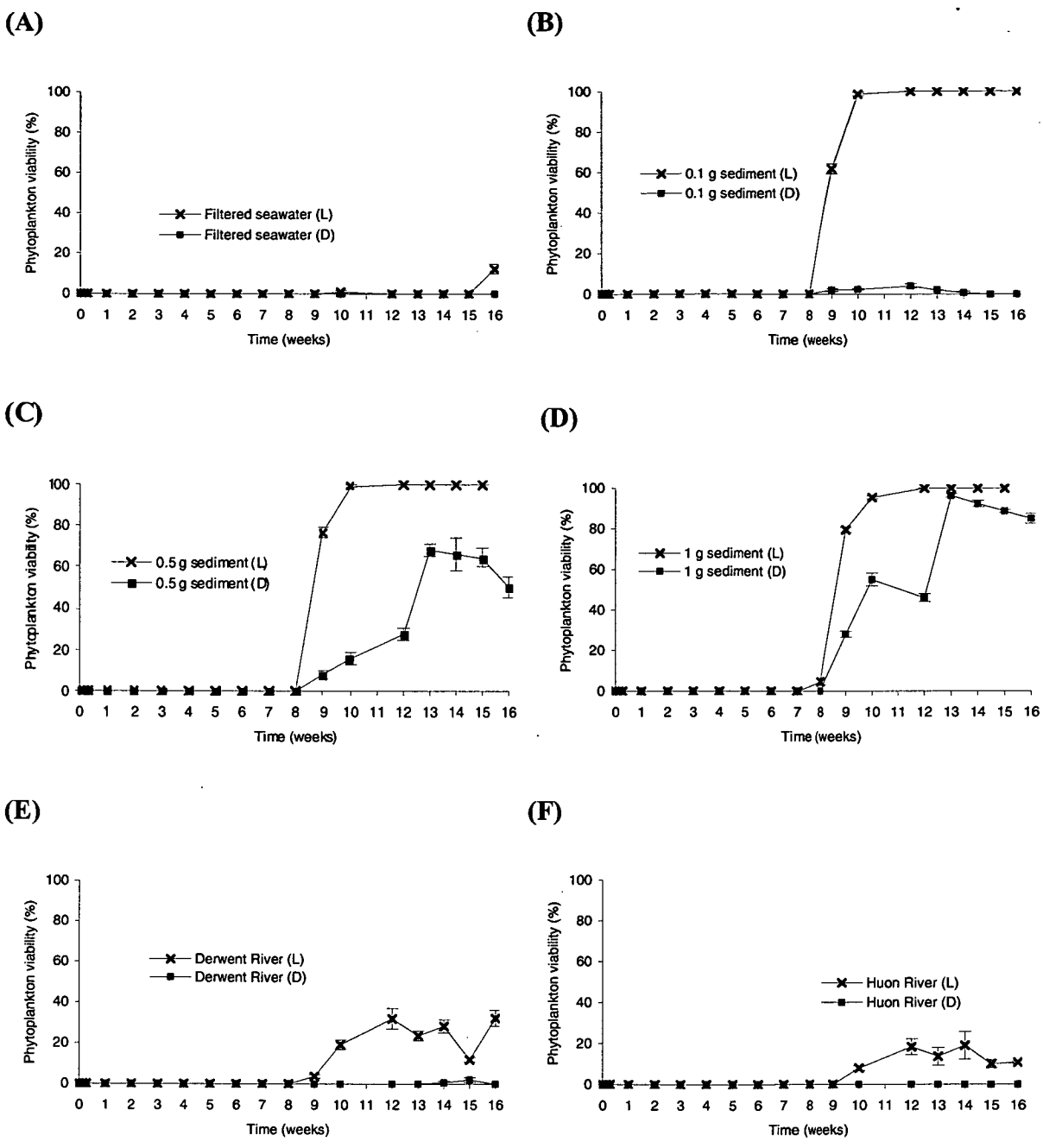


Fig. 16. Sensitivity of *Chattonella marina* to the ageing 10 ppm ECONEA® Technical samples. Assessed weekly by applying the ageing 10 ppm biocide concentrations to vegetative cells of the raphidophyte *Chattonella marina*. (**A**) in filtered seawater. (**B**) in filtered seawater containing 0.1 g of ballast sediment. (**C**) in filtered seawater containing 0.5 g of ballast sediment. (**D**) in filtered seawater containing 1 g of ballast sediment. (**E**) in seawater collected from the Derwent River. (**F**) in seawater collected from the Huon River. Samples were stored under both 12 light/12 h dark (L) and complete darkness (D).

3.5 Discussion

The biocide ECONEA® Technical was extremely effective at killing vegetative cells of both armoured and unarmoured dinoflagellates and raphidophytes. A concentration of 0.5 ppm was required for the complete inactivation of the six dinoflagellate and two raphidophyte species tested. Comparable efficacy data concerning the control of microalgae with ECONEA® Technical are limited. Preliminary trials conducted at the University of Newcastle upon Tyne found that ECONEA® was ineffective against two examined microalgal species, the silica-walled diatom *Cyclotella cryptica*, and the dinoflagellate *Alexandrium tamarense* (Tony Kempen, Janssen Pharmaceutica NV, pers. comm.). The latter University of Newcastle upon Tyne result is not consistent with the findings of the present work, as *Alexandrium tamarense* and the *Alexandrium* species used in the current study, *A. catenella*, are morphologically and genetically very closely related.

ECONEA® was not as effective at controlling vegetative cells of the small haptophyte *Prymnesium parvum* (8 ppm required) and did not completely inactivate the small cellulose-walled green-flagellate *Tetraselmis suecica* even at 20 ppm. This result may be due to the smaller cell size and greater cell densities of these species compared to the other microalgal species tested. The effect of cell size and density on the performance of ECONEA® requires further investigation. *Tetraselmis suecica* is not a harmful microalgae species however it is a good model organism for ballast water treatment as it is known to have a relatively thick cellulose cell wall and previous studies assessing the effectiveness of chemical biocides against this species have found it to be much more robust and difficult to kill compared to other phytoplankton species (Gregg and Hallegraeff, 2007).

ECONEA® failed to inactivate sexual resting cysts of the dinoflagellate *G. catenatum* at a concentration of 1000 ppm. This concentration is ten thousand times greater than that required to eliminate *G. catenatum* vegetative cells indicating that the product lacks the penetrability required to infiltrate the resistant cell walls of dinoflagellate cysts. In order to inactivate dinoflagellate cysts in ships ballast water, ECONEA® would require a co-biocide that is effective against cysts.

Out of the 14 potential co-biocides tested, several products were more effective than others at eliminating vegetative microalgae and dinoflagellate cysts. Natrium PYRION® 40% and PYRION® Disulfide 40% Suspension required the lowest concentrations for the effective control of vegetative cells and cysts. Natrium PYRION® 40% and PYRION® Disulfide 40% controlled the three microalgal test species at 0.1 ppm and 0.5 ppm, respectively, and inactivated *G. catenatum* resting cysts at 10 ppm. Biocides that could be disregarded as possible co-biocides to ECONEA® include Rimsulfuron PESTANAL®, Isoxaflutol PESTANAL® and LAg 2007 030 as they were not effective at eliminating the sensitive vegetative cells of *A. catenella* and failed to inactivate *G. catenatum* cysts. Although 4, 5-dichloro-N-octyl-4-isothiazolin-3-one was effective against vegetative microalgal cells at 60-100 ppm, the high concentration required to eliminate *G. catenatum* cysts (10 000 ppm) would also eliminate this product as a potential co-biocide. The remaining biocides could all effectively destroy *G. catenatum* cysts and vegetative cells of *A. catenella, P. parvum* and *T. suecica* at various concentrations; however the inactivation of the dinoflagellate cysts generally required considerably higher dosages of the chemicals. Some biocides were extremely effective against vegetative cells of *A. catenella* but required significantly higher dosages to inactivate vegetative cells of *P. parvum* and/or *T. suecica.* For example, PROTECTNOL BN could effectively control vegetative cells of *A. catenella* and *P. parvum* at a concentration of 2 ppm, and could control *G. catenatum* dinocysts at 25 ppm, yet the biocide did not cause the complete mortality of *T. suecica* vegetative cells at 20 ppm. Likewise, the Lag 2006 041 treatment eliminated *A. catenella* cells at 3 ppm but *P. parvum* and *T. suecica* required concentrations of 10 and 20 ppm, respectively.

Of the 14 possible co-biocides tested, products that have the potential include Hydrogen peroxide 30%, Natrium PYRION® 40%, Intercide OIT, PYRION® Disulfide 40% Suspension, KATHON™ 886F, ARQUAD MCB-50, PROTECTNOL BN, Oxy-PYRION® 98%, R090026 and LAg 2006. It should be highlighted that biological effectiveness alone is not an indicator of the suitability of the biocides or co-biocides. Other important factors that must be taken into consideration include the production cost of the chemicals as well as a variety of environmental and safety issues such as their degradability and environmental acceptability, the corrosive effects of the biocides on ships hulls and associated structures, and their ability to be

safely handled by crew members. Additional work is required to determine the effectiveness of each co-biocide combined with ECONEA® for controlling vegetative microaglal cells and dinoflagellate cysts.

Although ECONEA® Technical is suggested to rapidly degrade to non-detectable levels in seawater (half-lifes of 3 and 15 hours at 25 and 10°C, respectively) (ICES WGBOSV, 2006), one should not assume that non-detectable biocide levels represent non-toxic water. Therefore, the toxicity of treated ballast water is best assessed by using sensitive organisms in bioassay experiments. The results of the degradation experiments, using sensitive motile marine microalgae as bioassays, indicate that low concentrations of ECONEA® Technical (1 ppm) (i.e. the concentration required to eliminate dinoflagellate and raphidophyte vegetative cells) could degrade to a level non-toxic to marine microalgae in 4-6 weeks when kept under conditions of 12 h light/12 h dark at 17°C. ECONEA® Technical concentrations of 1 ppm that were kept in complete darkness were found to degrade at a considerably slower rate, requiring 8-12 weeks before samples were non-toxic to all four microalgal test species (*G. catenatum, A. catenella, P. reticulatum, C. marina*). This finding indicates that the degradation of ECONEA® Technical would be considerably slower inside ballast tanks compared to when the ballast water is discharged into the marine environment. One part-per-million ECONEA® Technical samples that contained ballast tank sediment always degraded faster than samples prepared in filtered seawater only. As the ballast tanks often contain significant accumulations of sediment, inorganic particles and organic detritus, this may act to promote the degradation of ECONEA® Technical.

Higher ECONEA® Technical concentrations (10 ppm) could only degrade to a level non-toxic to the four bioassay test species when samples contained ballast tank sediment and were exposed to light (after 16 weeks). Overall, seawater containing ballast tank sediment provided the best conditions for degradation. On the other hand, clean seawater, natural estuarine water, and a lack of light were the worst conditions for biodegradation. Overall, 10 ppm ECONEA® Technical concentrations prepared in natural estuarine water retained the highest level of toxicity following a period of 16 weeks. This would indicate that in a real ballasting situation, the treatment of water taken directly from ports with ECONEA® Technical may still

contain residual toxicants when the water is discharged back into the marine environment. The presence of ballast tank sediment, rust particles from tank corrosion and organic detritus may however act to accelerate the degradability of the product. Nonetheless, the discharge of ECONEA® Technical-treated ballast water has the potential to cause negative effects for the marine environment if ballast retention times are not sufficient to allow complete degradation to occur.

Results from the experiments assessing the degradation of ECONEA® Technical indicate that the compound degrades at a similar rate to that of the ballast water biocide Peraclean® Ocean. Comparable experiments conducted by Gregg and Hallegraeff (2007) on the degradability of the ballast water biocides SeaKleen® and Peraclean® Ocean found that low concentrations of Peraclean® Ocean (i.e. the concentration required to eliminate vegetative microalgal cells) degraded to a non-toxic level to marine microalgae in 2-6 weeks under 12 h light/12 h dark at 17°C; and degradation was also accelerated in the presence of sediments but reduced in the dark. ECONEA® Technical was found to degrade considerably faster than SeaKleen®. The degradation of low concentrations (4 ppm) of SeaKleen® was found to be minimal after 14 weeks and was not influenced by the presence of sediments, biological matter or light conditions.

The present work on the degradability of ECONEA® Technical found that certain microalgal species were considerably more sensitive to the residual toxicants or by-products formed during biocide degradation. The viability of the unarmoured dinoflagellate *G. catenatum* and the raphidophyte *C. marina* were significantly reduced compared to that of the armoured dinoflagellates *P. reticulatum* and *A. catenella* when exposed to degrading ECONEA® Technical samples. Therefore, the release of ECONEA® Technical-treated ballast water that has not completely degraded would have species-specific impacts on the microalgae at the point of discharge. Further work is required to aeess the degradability of ECONEA® when combined with potential co-biocides.

In summary, contrary to previous reports, ECONEA® was extremely effective against vegetative cells of dinoflagellates and raphidophytes. The biocide was less effective against the small haptophyte *P. parvum*, and was not effective against *T.*

suecica vegetative cells or dinoflagellate cysts of *G. catenatum.* Given this, for ECONEA® to be considered as a possible ballast water treatment option, it requires a co-biocide that is capable of eliminating the more resistant organisms. Potential co-biocides include Hydrogen peroxide 30%, Natrium PYRION® 40%, Intercide OIT, PYRION® Disulfide 40% Suspension, KATHON™ 886F, ARQUAD MCB-50, PROTECTNOL BN, Oxy-PYRION® 98%, R090026 and LAg 2006, however they need to be fully evaluated in combination with ECONEA® Technical. ECONEA® Technical was found to be degradable, especially when light and ballast tank sediments are present. However, caution should be taken to ensure that ECONEA® Technical-treated ballast water is retained in ballast tanks for a period long enough to allow adequate degradation to occur. There are several other issues regarding the suitability of ECONEA® (and the potential co-biocides) as a ballast water treatment option that require in-depth studies. These include: 1) the combined effectiveness against vegetative microalgae, dinoflagellate cysts and marine bacteria; 2) the influence of temperature on the biocidal activity of the chemicals; 3) the ability of ECONEA® (combined with the co-biocides) to inactivate organisms in sediment layers and in the presence of high sediment loads; 4) the degradability of ECONEA® combined with potential co-biocides and 5) the cost effectiveness of the chemical treatments.

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Chapter 4

Decreased ability of two commercially available ballast water biocides to inactivate dinoflagellate cysts when buried in ballast tank sediments

4.1 Abstract

Residual ship ballast sediment is known to harbour a variety of resting stages of potentially invasive organisms. The present study assessed the effectiveness of two commercially available ballast water biocides, Peraclean® Ocean and SeaKleen®, against resting cysts of the dinoflagellates *Gymnodinium catenatum* and *Alexandrium catenella* when buried in sediment from ballast tanks. In sediment-free treatments, effective control of *A. catenella* cysts was achieved at 200 ppm Peraclean® Ocean and 20 ppm SeaKleen®; and cysts of *G. catenatum* were killed at 1000 ppm Peraclean® Ocean and 8 ppm SeaKleen® following an exposure period of two weeks. The burial of cysts in 0.5 and 1mm deep ballast tank sediment, however, severely reduced the effectiveness of both chemical treatments. Complete inactivation of the *A. catenella* cysts was not achieved at 1000 ppm Peraclean® Ocean or 40 ppm SeaKleen® and a significant number of *G. catenatum* cysts were able to germinate following 2 weeks exposure to both 2000 ppm Peraclean® Ocean and 20 ppm SeaKleen®. The reduced efficacy of the biocides to inactivate dinoflagellate cysts buried in ballast tank sediment may be due to decreased diffusion rates of the chemicals into the sediment (notably with SeaKleen®) and/or accelerated degradation rates of the biocides in the presence of sediment (prevalent with Peraclean® Ocean).

Keywords: Sediment; Ballast water treatment; Chemical biocides; Dinoflagellate cysts

4.2 Introduction

Ships' ballast water is an established means for the dispersal of non-indigenous marine organisms around the globe (Minchin and Gollasch, 2002). When water is gravity fed or pumped into ships ballast tanks, any sediment (and associated biota) suspended in the water column is also taken on board. The intake and accumulation of sediment in ballast tanks can be significant and is a result of factors such as water depth and turbulence, wind and wave action, the type and function of the ship, the type and location of ballast tanks involved, port activities and the time since the tanks were last cleaned in dry dock (Hamer *et al.*, 2000; Hamer, 2002). Sediment accumulations varying from almost none to an estimated 100 tonnes per ships have been reported (Hallegraeff and Bolch, 1992; Hamer *et al.*, 2001). Recent studies have found diverse assemblages of organisms in tank sediments including numerous invertebrate species and their diapausing eggs (Bailey *et al.*, 2005a,b; Duggan *et al.*, 2006; Radziejewska *et al.*, 2006), microbial pathogens (Dobbs *et al.*, 2003) and resting stages of phytoplankton (Hallegraeff and Bolch, 1992; Hamer *et al.*, 2000, 2001). This indicates that ballast tank sediment provides a suitable habitat and may be an important means of dispersal for a wide range of organisms, including many potentially invasive species.

Of particular concern is the unintentional transfer of toxic dinoflagellates in the water and sediment of ships ballast tanks. Such species are capable of producing a range of toxins that can pose a threat to finfish and shellfish aquaculture, and can cause a number of human health issues (Hallegraeff, 1992). Most toxic dinoflagellates that affect aquaculture produce resistant sexual resting cysts that can remain viable in sediment for decades (Hallegraeff and Bolch, 1992; Lewis *et al.*, 1999). These resting stages are negatively buoyant, and thus readily accumulate on the bottom of ships' ballast tanks. The settling rate of cysts has been calculated to be much slower than that of similar sized inorganic particles indicating that cysts would generally be present in the upper layers of the sediment (Rigby and Hallegraeff, 2001). Cysts may originate both from resuspended port sediments and from dinoflagellate blooms in the water column at the time of ballasting (Hamer, 2002).

Dinoflagellate cysts are one of the most frequently found organisms in ships ballast water and tank sediments. A wide diversity of dinoflagellates cysts, including many toxic species, have been found in ballast tank sediments collected from ships entering ports in Australia (Hallegraeff and Bolch, 1992), New Zealand (Hay *et al.*, 1997), the United States (Kelly, 1993), Finland (Pertola *et al.*, 2006) and the United Kingdom (Hamer *et al.*, 2000, 2001). Cyst densities were found to vary considerably between ships and between ballast tanks of a single ship (Hamer *et al.* 2000, 2001). In one study, resting cysts were identified in 50% of the sediment containing samples, including high densities (up to 22,500/cm^2) of toxic *Alexandrium* cysts (Hallegraeff and Bolch, 1992).

At present, ballast water exchange is the only widely adopted management strategy for the control of organisms in ships ballast tanks. However, due to the dense nature of residual ballast water sediments and their tendency to accumulate in ballast tanks, ballast water exchange is unlikely to remove all ballast tank sediment. Additionally, ships that are laden with cargo carry only residual water and sediments in ballast tanks. These vessels are referred to as NOBOB (NO Ballast On Board) and are exempt from ballast water management practises as they are declared to contain no ballast water yet they may still carry significant amounts of un-pumpable water and accumulated sediment below the pump threshold (Duggan *et al.*, 2005). The amount of residual water and sediment in these ships is highly variable ranging from practically none to a reported 60-200 tonnes, of which sediments constitute up to 30% of total volume (Sano *et al.*, 2004; Duggan *et al.*, 2005). When a NOBOB vessel offloads its cargo, the tanks are filled with ballast water and the accumulated ballast tank sediment may be resuspended. This mixture of ballast water, residual water and sediment (and associated biota) is then discharged at subsequent ports-of-call. Given this, ships will require a treatment option that is capable of either effectively removing the ballast tank sediment (and associated organisms) or inactivating the organisms within the sediment.

Here we examine whether the potential ballast water biocides, Peraclean® Ocean and SeaKleen®, can inactivate sexual resting cysts of the toxic dinoflagellates *Gymnodinium catenatum* and *Alexandrium catenella* when buried in ballast tank sediment. Peraclean® Ocean and SeaKleen® have been demonstrated to kill a range of

ballast tank organisms including zooplankton (Fuchs and de Wilde, 2004; Sano *et al.*, 2004), phytoplankton (including resting stages) (Gregg and Hallegraeff, 2007) and bacteria (de Lafontaine *et al.*, 2007; Gregg and Hallegraeff, 2007). Both biocides have been recommended as possible treatment options for controlling sediment-dwelling organisms in NOBOB or unballasted vessels (Sano *et al.*, 2004; de Lafontaine *et al.*, 2008), yet the extent to which these biocides can eliminate organisms residing within the ballast tank sediment is unclear. Dinoflagellate cysts provide a useful model organism for testing the efficacy of NOBOB vessel or ballast tank sediment treatment options for several reasons. Apart from the regular incidence of dinoflagellate cysts in ballast tank sediment, and their ability to survive for long periods under unfavourable environmental conditions inside ballast tanks, cysts are extremely robust and thus any treatment option capable of inactivating dinoflagellate cysts in tank sediments would likely eliminate other sediment-dwelling ballast tank biota.

4.3 Materials and methods

Vegetative microalgal cultures

Vegetative cultures of the dinoflagellates *Gymnodinium catenatum* (strains GCDE11, GCTRA01) and *Alexandrium catenella* (strains ACTRA02, ACSHO2) were grown in 250 ml culture flasks containing 150 ml of GSe nutrient medium (Blackburn *et al.*, 1989). All cultured microalgal species were obtained from the microalgal culture collection at the School of Plant Science, University of Tasmania. Nutrient medium was made with filtered seawater collected from the Tasman Peninsula, Tasmania. Seawater was filtered using a 0.2μm membrane filter and was stored at 3°C. All cultures were maintained in a culture room at 17°C under 12h dark/ 12h light. Light was provided at an intensity of 100 μmol quanta $m^{-2}s^{-1}$ by a bank of cool-white fluorescent tubes.

Dinoflagellate cyst preparation

Dinoflagellate cysts of *Alexandrium catenella* were produced by inoculating two 2 ml culture suspensions of the compatible sexual mating strains ACTRA02 x ACSHO2

into 250 ml screw top beakers containing approximately 200 ml of filtered seawater (28‰ salinity). For *Gymnodinium catenatum* cysts, the compatible mating strains GCDE11 x GCTRA01 were inoculated into 250 ml screw top beakers containing 190 ml of filtered seawater and 10 ml of GSe medium. The screw top beakers were incubated at 17°C at a light intensity of 100 μmol quanta $m^{-2}s^{-1}$ and were examined at regular intervals for cyst formation. Once a workable quantity of cysts had formed, the screw top beakers were placed in the dark in order to kill the remaining vegetative cells. This was important for the *G. catenatum* experiments as the dead vegetative cell material sunk to the bottom of the screw top beakers and acted to agglutinate the cysts to the base of the beaker. This allowed the addition of the sediment without disturbing the position of the cysts. This was not as important for cysts of *A. catenella* as the mucoid coating surrounding the cyst wall firmly cemented them to the bottom of the screw top beakers. Prior to the addition of the sediment and biocides, the screw top beakers containing cysts were stored in the dark at a temperature of 4°C to minimise premature germination.

Chemical biocides

The stock solution of Peraclean® Ocean was a newly opened container containing a minimum of 14% Peracetic acid (Batch no. 24052004; Degussa Ag, Germany). For SeaKleen®, the stock solution was prepared from a freshly opened container of SeaKleen® 80 in the form of a wettable powder (Batch no. 041504; Garnett, Inc. Atlanta). All treatment solutions were prepared in filtered seawater (28‰ salinity).

Sediment and biocide application

The ballast tank sediment used in the current experiment was collected from the MV Princess Betty (No. 3 Port topside tank) in Geelong, Australia on 26-11-1989 and was stored at 4°C in the dark until use. Sediment was added to the cyst-containing beakers in two different quantities allowing the testing of each biocide to be carried out on cysts buried at depths of 0mm, and approximately 0.5 and 1mm. Sediment quantities of 0.55g and 1.1g were added to the screw top beakers to achieve the respective depths of 0.5 and 1 mm (based on the measured depth of five 10 g sediment samples). Prior to the addition of the sediment, the water volume in each screw top beaker was

carefully reduced to 50 ml using an Accu-jet® pipette equipped with a 50 ml pipette tip. For the addition of the sediment, quantities of 0.5 and 1g of ballast tank sediment were mixed with 50 ml of filtered seawater in 75 ml beakers. Sediment mixtures were sonicated for a duration of one minute to break up any large sediment flocs. The sediment suspensions were then carefully added to the cyst-containing beakers using an Accu-jet® pipette equipped with a 50 ml pipette tip. Following the addition of the sediment, the beakers were placed in the dark at 4°C for 2 days to minimise premature cyst germination and allow sedimentation. Once sedimentation had occurred the biocides were added. Peraclean® Ocean and SeaKleen® solutions were prepared in filtered seawater (28‰). A perforated polystyrene foam disc was placed on the water surface of each screw top beakers containing the sediment and cysts. This allowed the addition of the biocide solutions without disturbing the sediment layer. As the biocide solutions were added, the polystyrene disc floated on the water surface and was removed. For *G. catenatum* trials, Peraclean® Ocean was applied at concentrations of 0, 200, 600, 1000 and 2000 ppm, and SeaKleen® at concentrations of 0, 8, 15 and 20 ppm. *Alexandrium catenella* trials were exposed to biocide concentrations of 0, 200, 600 and 1000 ppm Peraclean® Ocean and 0, 8, 20 and 40 ppm SeaKleen®. Each biocide treatment and control was triplicated. Once the biocide solutions were added, all treatments were wrapped in aluminium foil and placed under culture conditions at 17°C for a period of 2 weeks.

Cyst collection and germination

Following 2 weeks exposure to the biocides, the cysts were removed from the trials. Cysts were removed and collected from treatments that contained sediment using sodium polytungstate (SOMETU®) as described by Bolch (1997). For trials containing no sediment, sodium polytungstate was not required for the collection of cysts. Cysts from the trials containing no sediment were collected using the following steps. Firstly approximately 150ml of the seawater was removed using an Accu-jet® pipette equipped with a 50 ml pipette tip. The remaining material was sonicated for approximately 1 min to dislodge the cysts from the bottom of the screw top beakers. Each sample was then passed through a 100-µm sieve and collected on a 25-µm sieve. The retrieved cysts were then resuspended in approximately 10 ml of filtered seawater and were allowed to settle. Cysts were then isolated by

micropipette, washed in fresh GSe medium in 35mm tissue culture dishes, and transferred to 12-well flat bottom microplates containing approximately 5ml of GSe medium. Microplates were sealed with parafilm, placed under culture conditions at 17°C exposed to a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ and a 12h dark/ 12h light photoperiod. Treatments were examined at weekly intervals for cyst germination using a Zeiss inverted microscope (Zeiss Axiovert 25).

Cysts were removed and concentrated from the treatments that contained ballast tank sediment using the following steps. One hundred and fifty millilitres of the seawater was removed using an Accu-jet® pipette equipped with a 50 ml pipette tip. The remaining material was sonicated for approximately 1 min to dislodge the cysts and homogenise the material. The samples were then passed through 100 µm sieves and collected on 25 µm sieves. The 25-100 µm fractions were resuspended in 20 ml of filtered seawater. Ten ml of the resuspended material was then added to two 15 ml screw-capped polyethylene centrifuge tubes for density separation using sodium polytungstate. Stock solutions of sodium polytungstate were prepared at a density of 2.4 g cm^{-2} by dissolving 150 g of sodium polytungstate in 50 ml of milli-Q water as described by Bolch (1997). Sodium polytungstate solutions were prepared at specific gravity of 1.4 g cm^{-2} by diluting the stock sodium polytungstate solution with milli-Q water. Four millilitres of the sodium polytungstate solution was carefully layered beneath the seawater/sediment suspensions using a glass pasteur pipette. The seawater/sediment suspensions were then centrifuged for 10 min at 3000 U/min.

Following centrifugation, the interface layer containing the cysts and organic material was removed using a glass pasteur pipette and placed in a 55 mm petri dish containing 10 ml of filtered seawater. Recovered cysts were then removed from this material by micropipette, washed in fresh GSe medium in 35mm tissue culture dishes, and transferred to 12-well flat bottom microplates containing approximately 5ml of GSe medium. Microplates were sealed with parafilm, placed under culture conditions at 17°C with a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ and a 12h dark/ 12h light photoperiod. Treatments were examined at weekly intervals for cyst germination using a Zeiss inverted microscope (Zeiss Axiovert 25).

4.4 Results

The results of current experiment assessing the ability of the two ballast water biocides to inactivate dinoflagellate cysts buried in ballast tank sediments are presented in Tables 1 and 2 for Peraclean® Ocean; and Tables 3 and 4 for SeaKleen®. Results show that burying cysts in ballast tank sediments at depths of approximately 0.5 and 1 mm does not cause a reduction in germination success for either *A. catenella* or *G. catenatum* (Tables 1-4).

Table 1. Effect of different concentrations Peraclean® Ocean on the germination success of resting cysts of the dinoflagellate *A. catenella* buried in ballast tank sediment (2 weeks exposure).

Biocide concentration (ppm)	Sediment depth (mm)	Viable cysts (viable\total)				Total cyst germination (%)
		Trial 1	Trial 2	Trial 3	Total	
0	0 (control)	27/36	20/30	18/22	65/88	73.8 ± 4.4
	0.5	51/52	22/30	19/25	92/107	86 ± 7.9
	1	16/19	27/31	37/44	80/94	85.1 ± 1
200	0 (control)	0/32	0/41	0/50	0/123	0
	0.5	27/36	29/35	31/51	87/122	71.3 ± 6.5*
	1	19/20	17/26	30/38	66/84	78.6 ± 8.6*
600	0 (control)	0/27	0/22	0/34	0/83	0
	0.5	10/17	25/35	17/29	52/81	64.2 ± 4.2*
	1	17/19	20/31	30/38	67/88	76.1 ± 7.2*
1000	0 (control)	0/17	0/29	0/37	0/83	0
	0.5	12/29	13/20	12/36	37/85	43.5 ± 9.5*
	1	12/21	16/30	19/31	47/82	57.3 ± 2.3*

* Denotes significant difference compared to control ($P<0.05$)

Peraclean® Ocean concentrations of 200, 600 and 1000 ppm were found to effectively inactivate all *A. catenella* cysts when treated in filtered seawater (control treatments) (Table 1). When *A. catenella* cysts were buried in ballast tank sediment at depths of 0.5 and 1 mm, however, cyst germination was evident in all trials at the three concentrations tested. Cyst germination success was the highest in the 1 mm trials compared to the 0.5 mm trials. Although germination success was found to

decline with an increase in biocide concentration, a significant number of cysts were still able to germinate at the maximum concentration tested. When exposed to 1000 ppm Peraclean® Ocean for a period of 2 weeks, 43.5 % and 57.3 % of *A. catenella* cysts were found to be viable when buried at the respective sediment depths of 0.5 and 1 mm (Table 1).

Table 2. Effect of different concentrations Peraclean® Ocean on the germination success of resting cysts of the dinoflagellate *G. catenatum* buried in ballast tank sediment (2 weeks exposure).

Biocide concentration (ppm)	Sediment depth (mm)	Viable cysts (viable\total) Trial 1	Trial 2	Trial 3	Total	Total cyst germination (%)
0	0 (control)	38/41	43/46	36/41	117/128	91.4 ± 1.8
	0.5	40/47	32/38	54/59	126/144	87.5 ± 2.3
	1	50/55	35/42	34/39	119/136	87.5 ± 2.2
200	0 (control)	36/38	32/38	35/40	103/116	88.8 ± 3.1
	0.5	48/56	30/35	41/44	119/135	88.1 ± 2.5
	1	32/37	40/50	28/34	100/121	82.6 ± 1.9
600	0 (control)	24/40	14/32	17/49	55/121	45.5 ± 7.4
	0.5	20/29	31/43	26/33	77/105	73.3 ± 2.9*
	1	37/47	27/39	20/29	84/115	73 ± 3.2*
1000	0 (control)	0/51	0/37	0/39	0/127	0
	0.5	19/25	30/36	27/35	76/96	79.2 ± 2.3*
	1	51/70	13/18	11/17	75/105	71.4 ± 2.6*
2000	0 (control)	0/37	0/22	0/28	0/87	0
	0.5	8/22	19/40	21/39	48/101	47.5 ± 5.1*
	1	22/28	16/32	20/31	58/91	63.7 ± 8.3*

* Denotes significant difference compared to control ($P<0.05$)

Gymnodinium catenatum cysts were found to be more resistant to the Peraclean® Ocean treatment compared to those of *A. catenella.* A higher Peraclean® Ocean concentration of 1000 ppm was required for the complete inactivation of *G. catenatum* cysts when treated in filtered seawater with no sediment (Table 2). Burying the cysts in sediment caused a significant increase in the germination success of *G. catenatum* in the 600, 1000 and 2000 ppm treatments (Table 2). When

buried at a depth of 0.5 mm, 47.5% of cysts were found to be viable following 2 weeks exposure to 2000 ppm Peraclean® Ocean (Table 2). Increasing the sediment depth to 1 mm further reduced the effectiveness of the biocide.

The effectiveness of SeaKleen® was also found to be limited when the dinoflagellate cysts were covered by ballast tank sediment. When *A. catenella* cysts were exposed to SeaKleen® at a concentration of 8 ppm for 2 weeks, only 56.5% of cysts were found to be viable when no sediment was present compared to 72% and 83.5 % in the 0.5 and 1 mm depth trials (Table 3). In the absence of sediment, resting cysts of *A. catenella* were completely inactivated at a SeaKleen® concentration of 20 ppm following 2 weeks exposure, however, when exposed to *A. catenella* cysts buried in sediment at depths of 0.5 and 1 mm, complete inactivation did not occur at concentrations of either 20 and 40 ppm (Table 3).

Table 3. Effect of different concentrations of SeaKleen® on the germination success of resting cysts of the dinoflagellate *A. catenella* buried in ballast tank sediment (2 weeks exposure).

Biocide concentration (ppm)	Sediment depth (mm)	Viable cysts (viable\total)				Total cyst germination (%)
		Trial 1	Trial 2	Trial 3	Total	
0	0 (control)	30/37	42/50	31/36	103/123	83.7 ± 1.4
	0.5	21/27	29/29	19/22	69/78	88.5 ± 6.5
	1	50/59	48/57	21/26	119/142	83.8 ± 1.2
8	0 (control)	9/15	23/39	20/38	52/92	56.5 ± 2.3
	0.5	16/25	18/25	33/43	67/93	72 ± 3.7*
	1	22/27	20/23	29/35	71/85	83.5 ± 1.6*
20	0 (control)	0/19	0/27	0/33	0/69	0
	0.5	14/28	12/29	17/37	43/94	45.7 ± 2.4*
	1	22/47	19/29	22/30	63/106	59.4 ± 7.9*
40	0 (control)	0/37	0/41	0/20	0/98	0
	0.5	9/32	11/33	19/47	39/112	34.8 ± 3.6*
	1	16/30	19/32	12/22	47/84	55.9 ± 1.9*

* Denotes significant difference compared to control ($P<0.05$)

Complete inactivation of *G. catenatum* cysts was achieved at 15 ppm when exposed to the biocide for 2 weeks in filtered seawater (Table 4). Yet again, the addition of ballast tank sediment severely reduced the effectiveness of the biocide. SeaKleen® failed to completely inactive *G. catenatum* cysts at both 15 and 20 ppm (Table 4). Although the germination success of the sediment trials in the 20 ppm SeaKleen® treatment were considerably lower than values obtained in the sediment trials that were not treated, total cyst germination was still over 50% for both sediment depths tested (Table 4).

Table 4. Effect of different concentrations of SeaKleen® on the germination success of resting cysts of the dinoflagellate *G. catenatum* buried in ballast tank sediment (2 weeks exposure).

Biocide concentration (ppm)	Sediment depth (mm)	Viable cysts (viable\total)				Total cyst germination (%)
		Trial 1	Trial 2	Trial 3	Total	
0	0 (control)	58/65	51/63	42/45	151/173	87.3 ± 3.6
	0.5	37/42	50/57	47/57	134/156	85.9 ± 1.8
	1	22/31	17/24	43/56	82/111	73.9 ± 2
8	0 (control)	32/63	40/69	37/65	109/197	55.3 ± 2.8
	0.5	29/38	36/44	16/24	81/106	76.4 ± 4.4*
	1	22/28	50/62	34/49	106/139	76.3 ± 3.4*
15	0 (control)	0/30	0/39	0/44	0/113	0
	0.5	24/33	32/44	19/30	75/107	70.1 ± 3.1*
	1	37/50	26/35	35/49	98/134	73.1 ± 0.9*
20	0 (control)	0/47	0/32	0/41	0/120	0
	0.5	16/42	14/23	27/40	57/105	54.3 ± 8.9*
	1	22/39	26/44	21/50	69/133	51.9 ± 5.3*

* Denotes significant difference compared to control ($P<0.05$)

4.5 Discussion

The control of sediment-dwelling organisms in ships ballast tanks has received relatively little attention compared to free-living and motile organisms in ballast water. As ballast tank sediment has been documented to contain a variety of toxic and potentially invasive species, in order for a ballast water treatment option to be

successful, it must not only control ballast water organisms but also those contained within the ballast tank sediment. The two biocides tested in the present study were unable to inactivate sexual resting cysts of the dinoflagellates *Alexandrium catenella* and *Gymnodinium catenatum* when buried in ballast tank sediment.

The burial of dinoflagellate cysts in ballast tank sediment significantly reduced the efficacy of both SeaKleen® and Peraclean® Ocean. This result is consistent with previous studies on the impact of sediment on the biocidal efficacy of SeaKleen® (Sano *et al.*, 2004; Raikow *et al.*, 2006) and Peraclean® Ocean (de Lafontaine *et al.*, 2008).

Raikow *et al.* (2006) tested the efficacy of SeaKleen® against a variety of invertebrate resting stages and found that the burial of *Daphnia mendotae* ephippia in natural lake sediment at depths of up to 1 cm reduced SeaKleen® toxicity by a factor of 20. In the present experiment, SeaKleen® and Peraclean® Ocean failed to eliminate dinoflagellate cysts buried in sediment at depths of only 0.5 and 1mm. As sediment accumulations ranging from almost none to depths of over 30 cm have been recorded in the bottoms of ballast tanks (Hamer *et al.*, 2001), the use of these chemical biocides would be unlikely to inactivate invertebrate resting stages buried in sediment retained in ships ballast tanks.

Raikow *et al.* (2006) suggest that the likely mechanism of the observed protective effect of the sediments on the toxicity of chemical biocides is decreased exposure due to low diffusion rates of the chemicals into the sediment. In addition to decreased exposure, the limited ability of the biocides to destroy the dinoflagellate cysts in the ballast tank sediment may also be caused by accelerated degradation rates of the chemicals in the presence of sediment and organic detritus. De Lafontaine *et al.* (2008) revealed that the presence of sediment and biological matter, together with rust and other metal particles resulting from tank corrosion, acted to increase the degradation rate of Peraclean® Ocean. Gregg and Hallegraeff (2007) also found that the toxicity of Peraclean® Ocean to vegetative microalgae was reduced when ballast tank sediment was present. It is likely that the reduced toxicity of Peraclean® Ocean demonstrated in the present study is due to a combination of the factors mentioned above.

Previous work indicated that the toxicity of SeaKleen® was not affected by the presence of sediment (Gregg and Hallegraeff, 2007). Therefore, the failure of SeaKleen® to inactivate dinoflagellate cysts buried in ballast tank sediment is more likely caused by reduced exposure to the chemical due the protective effect of the sediment layer rather than the loss of toxicity due to the presence of sediment. Similarly, in studies by Sano *et al.* (2004) on the amphipod *Hyalella azteca,* control was achieved at comparable SeaKleen® concentrations in both sediment-free samples (LC90=2.5 ppm) and samples containing a 1:4 sediment to water ratio (LC90=3.5 ppm), whereas the LC90 value for the burrowing oligochaete *Lumbriculus variegates* was 49 times higher in the 1:4 sediment to water ratio (88 ppm) compared to the water-only exposures (1.8 ppm).

While effective control of sediment-dwelling organisms still might be possible using much higher concentrations of Peraclean® Ocean and SeaKleen® than used in the present study, the use of either chemical is likely to be prohibitively expensive and would require dosages that would pose environmental problems due to the discharge of toxic ballast water and residual sediment.

As sediment-dwelling organisms in ships ballast tanks are likely to prove difficult to eliminate with chemical biocides, minimising sediment uptake during ballast intake or removing the sediments by regular in-port cleaning or redesigning ballast tank configurations would be required to reduce the risk of introducing non-indigenous organisms into new areas with the discharge of residual ballast water and tank sediment. Management practises that can act to reduce the amount of sediment (and associated organisms) taken onboard include avoiding ballasting in shallow areas, areas of high water turbidity, and in regions that are subject to port dredging activities. Ship operators should also avoid ballasting in areas during toxic dinoflagellate bloom events, especially during periods when resting cysts are present in the water column (Hallegraeff, 1998).

Filtration and cyclonic separation are commercially available treatment options that aim to remove suspended matter at the point of ballast water uptake. At present, these types of systems have been demonstrated to be operationally efficient at

removing particles down to a size of 50 microns (e.g. Cangelosi *et al.*, 2007). This may effectively remove some large dinoflagellate cyst species, as well as larger sediment flocs, gravel and sand particles, but clay, silt and small plankton (<50μm) will be largely unaffected. Heat treatment has been demonstrated as an effective tool for destroying dinoflagellate cysts (Hallegraeff *et al.*, 1997), and has been suggested as a possible treatment option for NOBOB vessels (Stocks *et al.*, 2004). Previous assessments of the use of heat to treat ballast tank residues revealed an uneven distribution of heated water between and within ballast tanks (temperature rises from 15-40°C) due to factors such as tank configuration, distance from hot water input, location of circulating pumps and amount of sediment in ballast tanks (Stocks *et al.*, 2004). Rigby and Taylor (2001) indicate that higher temperatures are experienced at the bottom of tanks. In their study, the heated ballast water was pumped into this region indicating that the heating of ballast water has the potential to effectively treat organisms present in ballast sediment. The use of heat for controlling sediment-dwelling organisms in ships ballast tanks requires further investigation.

Ideally, a ballast tank should be designed to ensure good sediment removal when discharging ballast water, with only a minimal retention of sediments in the tank. Current IMO guidelines list a range of design concepts that should be taken into consideration to avoid the accumulation of sediment in ballast tanks and their internal structures. These include: 1) avoiding horizontal surfaces wherever possible; 2) incorporating extra drainage holes in internal ballast tank structures which allows water to flow with minimal restriction during discharge; 3) pipeline systems should be designed to create the maximum water disturbance possible when deballasting to resuspend and discharge sediment; and 4) ship design should provide full access to ballast tanks for sediment removal (MEPC, 2006).

In summary, the limited effectiveness of SeaKleen® and Peraclean® Ocean against dinoflagellate cysts buried in sediments raises serious questions for the use of these chemical biocides for the purpose of eliminating organisms in ships ballast tank sediment. Current vessel and future ship designs should aim to minimise the uptake, entrapment, accumulation and retention time of sediments in ballast tanks.

4.6 References

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Chapter 5

Validation of Sytox® Green fluorescent staining for assessment of dinoflagellate cyst viability

5.1 Abstract

The fluorescent nucleic acid stain Sytox® Green, which does not penetrate living cells, was used in conjunction with fluorescent microscopy to determine the viability of dinoflagellate cysts following treatment with the ballast water chemical biocides Peraclean® Ocean and SeaKleen®. The method was validated against conventional cyst germination experiments. In the Sytox® Green staining experiments, and similarly in the parallel germination experiments, temporary cysts of *Alexandrium pseudogonyaulax* and resting cysts of *Alexandrium catenella* and *Protoceratium reticulatum* were not viable at concentrations of 600 ppm Peraclean® Ocean, and 6, 20 and 10 ppm of SeaKleen®, respectively, and no viable *Gymnodinium catenatum* resting cysts were evident in the 2000 ppm Peraclean® Ocean or 20 ppm SeaKleen® treatments after 2 weeks exposure. The fluorescent stain was capable of infiltrating the compromised cell wall of dead cysts causing them to fluoresce bright green when exposed to a 450-490 nm light source. At sub-lethal biocide concentrations, total cyst viability was always found to be higher in the Sytox® Green viability staining experiments suggesting that the 6-week observation period used in the germination experiments may not have been sufficient for all viable cysts to germinate. Dead *G. catenatum* cysts did not fluoresce as well as the other test species, except in cases where the brown-pigmented cyst wall was completely bleached or removed, nonetheless, live and dead cells were still distinguishable. Some variability in the viability of different batches of *G. catenatum* cysts was also noted. The major advantage of the Sytox® Green method is that cyst viability can be determined in several hours compared to the >6 weeks required using the conventional germination method.

Keywords: Dinoflagellate cysts; Ballast water treatment; Viability; Sytox® Green

5.2 Introduction

One possible factor contributing to the apparent global spreading of toxic dinoflagellates is the transport of such species in ships' ballast water and sediment (Hallegraeff and Bolch, 1991). Several toxic dinoflagellates are of specific concern in relation to ballast water mediated dispersal due to the development of resistant resting cysts. Dinoflagellate resting cysts are formed under adverse environmental conditions and readily accumulate in ballast tank sediment from which they are not always effectively removed by ballast water exchange (Hallegraeff and Bolch, 1992; Doblin and Dobbs, 2006). Many cases exist whereby large quantities of cysts, including toxic species, have been discovered in ships ballast tanks (e.g. Hallegraeff and Bolch, 1992; Kelly, 1993; Hay *et al.,* 1997; Hamer *et al.,* 2000, 2001; Pertola *et al.,* 2006). Cysts can survive for years in ballast tanks and in port sediments following ballast water discharge. Therefore, dinoflagellate cysts are one of the most well studied organisms in investigations of risks associated with the introduction of non-indigenous organisms in ballast water and have been suggested as a possible surrogate organism to evaluate the efficacy of ballast water treatments (Matheickal *et al.,* 2004). Dinoflagellate resting cysts provide a good model organism for the assessment of ballast water treatment options as they are extremely robust and have been demonstrated to be resistant to several sterilisation techniques including UV irradiation (Oemcke, 1999; Valentine, 1996; Montani *et al.,* 1995) and a variety of chemical biocides (Bolch and Hallegraeff, 1993; Ichikawa *et al.,* 1993; Montani *et al.,* 1995). It can therefore be argued that a treatment system capable of killing dinoflagellate cysts will likely kill a wide range of other target organisms that occur in ballast water and sediments (Hallegraeff, 1998).

The use of dinoflagellate cysts for assessing the efficacy of ballast water treatment options has been limited due to the time required to determine cyst viability following exposure to potential treatment options. Conventional methods used for the determination of cyst viability have included the presence of a healthy swimming planomeiocyte and the incidence of an empty cyst wall (Ichikawa *et al.,* 1992; Bolch and Hallgraeff, 1993; Hallegraeff *et al.,* 1997). These techniques involve careful microscopic observations of cyst gemination for a period of up to several months as the cysts of many species must undergo a mandatory dormancy period before they are

physiologically capable of germination. The duration of this process is highly variable among species, and within a single species, ranging from several hours to 12 months (Hallegraeff *et al.,* 1998; Perez *et al.,* 1998; Kremp and Anderson, 2000), thus limiting the use of dinoflagellate cysts for assessing ballast water treatments to species that have a short dormancy period. Given this, a procedure for the rapid determination of dinoflagellate cyst viability would prove valuable for both the assessment of cyst viability following ballast water treatments and the determination of cyst viability in ships ballast tanks and port sediments.

The present study examines the effectiveness of the nucleic acid stain, Sytox® Green, as a tool for the rapid assessment of dinoflagellate cyst viability. Sytox® Green is a fluorescent viability stain that penetrates cells with compromised cell membranes yet will not cross the membranes of live cells (Molecular Probes, 2001). It has been used to assess the viability of bacterial cells (Roth *et al.,* 1997; Lebaron, *et al.,* 1998), phytoplankton (Veldhuis *et al.,* 1997; Binet and Stauber, 2006; Veldhuis *et al.,* 2006), copepods (Buttino *et al.,* 2004) and nematodes (Gill *et al.,* 2003). Binet and Stauber (2006) successfully used Sytox® Green in conjunction with flow cytometry for the determination of dinoflagellate cyst viability in less than 2 days after treatment, however, this study was limited to a single test species (*Alexandrium catenella*). The present experiment builds on the work of Binet and Stauber (2006) by examining the reliability of Sytox® Green for determining dinoflagellate cyst viability using fluorescent microscopy. The validity of the technique was determined by conducting parallel germination experiments following the treatment of resting cysts of the dinoflagellates *Alexandrium catenella, Gymnodinum catenatum* and *Protoceratium reticulatum* and temporary cysts of *Alexandrium pseudogonyaulax* with the ballast water biocides Peraclean® Ocean and SeaKleen®.

5.3 Materials and methods

Vegetative microalgal cultures

Vegetative cultures of the dinoflagellates *Gymnodinium catenatum, Alexandrium catenella, Alexandrium pseudogonyaulax* and *Protoceratium reticulatum* were grown in 250 ml culture flasks containing 150 ml of GSe nutrient medium (Blackburn *et al.,*

1989). Table 1 summarises isolation details of the microalgal strains used.). All cultured microalgal species were obtained from the microalgal culture collection at the School of Plant Science, University of Tasmania. Nutrient medium was made with filtered seawater collected from the Tasman Peninsula, Tasmania. Seawater was filtered using a 0.2μm membrane filter and was stored at 3°C. All cultures were maintained in a culture room at 17°C under 12h dark/ 12h light. Light was provided at an intensity of 100 μmol quanta $m^{-2}s^{-1}$ by a bank of cool-white fluorescent tubes.

Table 1. Isolation details and characteristics of microalgal strains used.

Species	Culture code	Date of isolation	Source	Isolated by	Status
A. catenella	ACTRA02	17/10/1997	Triabunna (TAS)	C. Bolch	Clonal
A. catenella	ACSH02	N/A	Sydney Harbour (NSW)	S. Norwood	N/A
A. pseudogonyaulax	APGB02	2006	Georges Bay (TAS)	M. de Salas	N/A
G. catenatum	GCDE11	N/A	Derwent River (TAS)	M. de Salas	Unialgal
G. catenatum	GCTRA01	24/05/2000	Triabunna (TAS)	M. de Salas	Clonal
P. reticulatum	PTRDE04	15/05/2000	Derwent River (TAS)	M. de Salas	Unialgal
P. reticulatum	PTRDE11	1999	Derwent River (TAS)	N. Parker	Clonal

Dinoflagellate cyst production

Resting cysts of the dinoflagellates *Alexandrium catenella* and *Protoceratium reticulatum* were produced by inoculating 2 ml culture suspensions of compatible sexual mating strains (strain ACTRA02 x ACSH02 and PTRDE04 x PTRDE11) into 250 ml screw top beakers containing 200 ml of filtered seawater (28‰ salinity). For *G. catenatum*, cysts were produced by inoculating 2 ml suspensions of the compatible sexual mating strains GCDE11 and GCTRA01 into 250 ml screw top beakers containing 180 ml of filtered seawater (28‰ salinity) with 20 ml of GSe medium. The vegetative *A. pseudogonyaulax* culture (APGB02) produced temporary cysts in 250 ml screw top beakers containing 200 ml of GSe nutrient medium. The screw top beakers were incubated at 17°C at a light intensity of 100 μmol quanta $m^{-2}s^{-1}$ and were examined at regular intervals for cyst formation. This procedure produced large numbers of cysts within 28 days for *G.catenatum* (approximately 1000-2000 per beaker) and within 21 days for *A. catenella* (2000-4000 per beaker)

and *P. reticulatum* (approximately 5000 per beaker) of which 80 to 95% readily germinated within 6 weeks. *Alexandrium pseudogonyaulax* cultures immediately started to produce cysts. After 14 days, approximately 2000 cysts were present in each beaker. The germination success of *A. pseudogonyaulax* cysts was difficult to quantify as cysts were constantly germinating and forming. *Alexandrium catenella* cysts were subjected to a 17-25°C temperature change for 24 h to induce germination (after Hallegraeff *et al.*, 1998). Following this stimulation, germination occurred after 7 days. Prior to treatment, all cysts were stored at 4°C in the dark to prevent premature germination.

Chemical biocides

The stock solution of Peraclean® Ocean was a newly opened 10 L container of Peraclean® Ocean 150 containing 15% peroxyacetic acid (Batch no. 32953141566; Degussa Peroxide Limited, New Zealand). For SeaKleen®, the stock solution was prepared from a freshly opened container of SeaKleen® 80 in the form of a wettable powder (Batch no. 041504; Garnett, Inc. Atlanta). All treatment solutions were prepared in filtered seawater (28‰ salinity).

Chemical treatment of dinoflagellate cysts

Dinoflagellate cysts were placed into 12-well flat bottom microplates containing 4 ml of GSe medium and various concentrations of the biocides were subsequently applied. *Gymnodinium catenatum* cysts were exposed to concentrations of 600, 1000 and 2000 ppm Peraclean® Ocean, and 10 and 20 ppm of SeaKleen®. *Alexandrium catenella* cysts were exposed to 600 and 1000 ppm of Peraclean® Ocean, and 20 and 40 ppm of SeaKleen®. *Protoceratium reticulatum* cysts were exposed to 600 and 1000 ppm of Peraclean® Ocean and 10 and 20 ppm of SeaKleen®; and For *A. pseudogonyaulax*, cysts were exposed to concentrations of 100 and 600 ppm Peraclean® Ocean, and 6 and 12 ppm of SeaKleen®. Each concentration and control was replicated 3 times. After the application of the biocide, the culture plates were sealed with parafilm, wrapped in aluminium foil and placed under culture conditions at 17°C for a period of 2 weeks.

Germination experiment

After 2 weeks exposure to the chemical biocides, treatments were sonicated for 30 seconds to dislodge the cysts. Approximately 20-50 individual cysts from each trial were removed, washed in sterile GSe, and transferred to 12-well flat bottom microplates containing fresh GSe media. The treated cysts were then placed back under culture conditions with a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ at 17°C with a 12h dark/ 12h light photoperiod. Treatments were examined at weekly intervals for cyst germination for a period of 6 weeks. The existence of a healthy swimming planomeiocyte and the incidence of an empty cyst wall were used as criteria for successful cyst germination. Germination observations were conducted on a Zeiss inverted microscope (Zeiss Axiovert 25).

Sytox® Green viability staining

After 2 weeks exposure to the chemical biocides, samples were sonicated for 30 seconds to dislodge cysts. Cysts were then removed using a micropipette, washed in sterile GSe and placed into 55 mm petri dishes containing 5 ml of Milli-Q water. Two microlitres of Sytox® Green was applied to the dinoflagellate cysts and each petri dish was incubated for 60 min at 17°C in the dark. Following incubation, cysts were transferred to a microscope slide using a micropipette and viability was determined using a Zeiss Axioskop fluorescent microscope equipped with a 450-490 fluorescent light source (excitation wavelength 450-490 nm, emission wavelength 515 nm). Sytox® Green binds to the nucleic acids of dead cells causing them to fluoresce green when excited by a 450-490 nm light source.

5.4 Results

A comparison of the results obtained in the germination and Sytox® Green viability staining experiments shows an excellent agreement for the biocide concentrations required to inactivate dinoflagellate cysts. In the Sytox® Green staining experiments, and similarly in the parallel germination experiments, cysts of the dinoflagellates *A. catenella, A. pseudogonyaulax* and *P. reticulatum* were not viable following 2 weeks exposure to biocide concentrations of 600 ppm Peraclean® Ocean, and 20, 6 and 10

ppm of SeaKleen®, respectively, and no viable *G. catenatum* cysts were evident in the 2000 ppm Peraclean® Ocean or 20 ppm SeaKleen® treatments (Tables 2 and 3).

At sub-lethal biocide concentrations, total cyst viability was always found to be higher in the viability staining experiments compared to the germination experiments. For example, following 2 weeks exposure to a Peraclean® Ocean concentration of 1000 ppm, *G. catenatum* cyst viability was 60.9% in the Sytox® Green staining experiment compared to 15.1% in the germination experiment (Table 2). Likewise, when exposed to 10 ppm of SeaKleen®, 12.9% of *G. catenatum* cysts were found to be viable when stained with Sytox® Green, while only 2.6% of cysts germinated (Table 3) following an exposure period of 2 weeks.

Table 2. A comparison of the germination success and viability determined by Sytox® Green of cysts of the dinoflagellates of *A. catenella, A. pseudogonyaulax, G. catenatum* and *P. reticulatum* after 2 weeks exposure to different concentrations of Peraclean® Ocean (as % of total number of cysts used).

Peraclean® Ocean concentration (ppm)	Total cyst germination (%)	Total cyst viability (%) (Sytox® Green viability experiment)
Alexandrium catenella		
0 (control)	89 ± 3.1 (73)	96.3 ± 3.3 (27)
600	0* (77)	0* (37)
1000	0* (81)	0* (40)
Alexandrium pseudogonyaulax		
0 (control)	61.4 ± 4.9 (83)	93.8 ± 4.4 (32)
100	58.1 ± 6.6 (74)	71.6 ± 3.1* (67)
600	0* (80)	0* (31)
Gymnodinium catenatum		
0 (control)	95 ± 2.5 (119)	94.6 ± 3.2 (37)
600	89.4 ± 2 (113)	92.3 ± 3.7 (39)
1000	15.1 ± 5.8* (139)	60.9 ± 1.7* (46)
2000	0* (102)	0* (37)
Protoceratium reticulatum		
0 (control)	89.7 ± 3.7 (88)	100 (51)
600	0* (96)	0* (57)
1000	0* (93)	0* (39)

The numbers in parentheses are the total number of cysts treated. * Denotes significant difference compared to control ($P<0.05$).

Table 3. A comparison of the germination success and viability determined by Sytox® Green of cysts of the dinoflagellates of *A. catenella, A. pseudogonyaulax, G. catenatum* and *P. reticulatum* after 2 weeks exposure to different concentrations of SeaKleen® (as % of total number of cysts used).

SeaKleen® concentration (ppm)	Total cyst germination (%)	Total cyst viability (%) (Sytox® Green viability staining)
Alexandrium catenella		
0 (control)	87 ± 5.8 (77)	97.8 ± 2.2 (45)
20	0* (85)	0* (24)
40	0* (89)	0* (25)
Alexandrium pseudogonyaulax		
0 (control)	63.6 ± 12.8 (66)	97 ± 2.6 (33)
6	0* (78)	0* (26)
12	0* (62)	0* (28)
Gymnodinium catenatum		
0 (control)	91.3 ± 2.8 (103)	100 (34)
10	2.6 ± 2.7* (75)	12.9 ± 5.9* (31)
20	0* (92)	0* (26)
Protoceratium reticulatum		
0 (control)	83.8 ± 3 (80)	95.6 ± 3.2 (45)
10	0* (101)	0* (31)
20	0* (74)	0* (34)

The numbers in parentheses are the total number of cysts treated. * Denotes significant difference compared to control ($P<0.05$).

Figures 1 and 2 show the fluorescent properties of treated (dead) and untreated (live) dinoflagellate cysts stained with Sytox® Green. The stain failed to penetrate the cell wall of live cysts (Fig.1) but infiltrated the compromised cell wall of dead cysts causing them to fluoresce green when exposed to the 450 nm light source (Fig. 2).

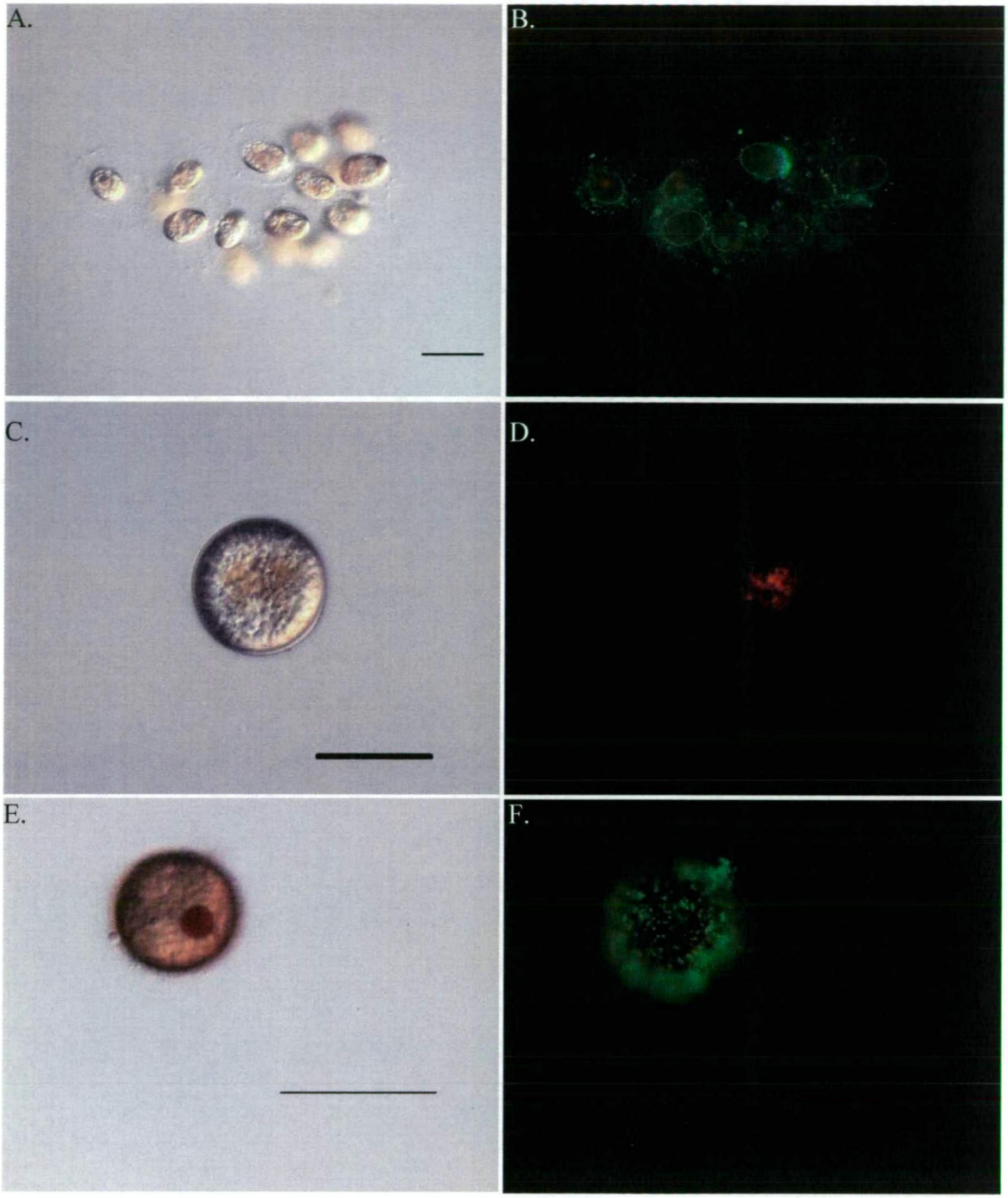

Fig. 1. Fluorescence of untreated cysts stained with Sytox® Green showing the failure of the stain to penetrate intact cyst. (A) Control *A. catenella* cysts; (B) control *A. catenella* cysts viewed under fluorescent microscope (450-490 excitation wavelength, 515 emission wavelength); (C) control *A. pseudogonyaulax* cyst; (D) control *A. pseudogonyaulax* cyst viewed under fluorescent microscope (450-490 excitation wavelength, 515 emission wavelength); (E) control *G. catenatum* cyst; (F) control *G. catenatum* cyst view under fluorescent microscope (450-490 excitation wavelength, 515 emission wavelength). Scale bars=50μm

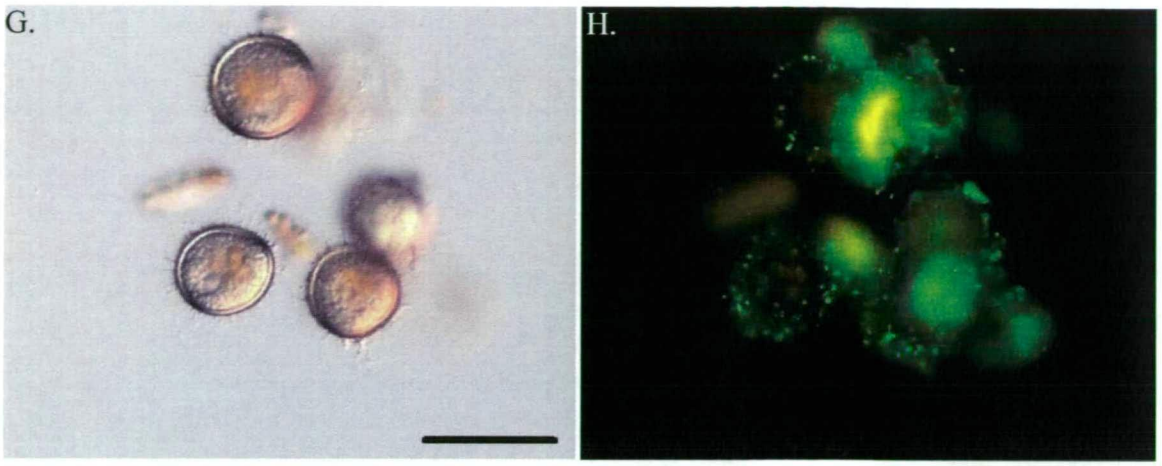

Fig. 1 *cont.*. Fluorescence of untreated cysts stained with Sytox® Green showing the failure of the stain to penetrate intact cyst. (G) Control *P. reticulatum* cysts; (H) control *P. reticulatum* cysts view under fluorescent microscope (450-490 excitation wavelength, 515 emission wavelength). Scale bars=50μm

The inability of the stain to penetrate viable *A. catenella, G. catenatum* and *P. reticulatum* cysts can be seen in Figure 1B, F and H, whereby the stain binds to the nucleic acids of organic material attached to the exterior of the cysts, but fails to penetrate the cyst walls. Dead *G. catenatum* cysts did not fluoresce as brightly as the other test species (Fig. 2E, F), except when the brown-pigmented sporopollenin cyst wall was completely bleached or removed (Fig. 2G, H).

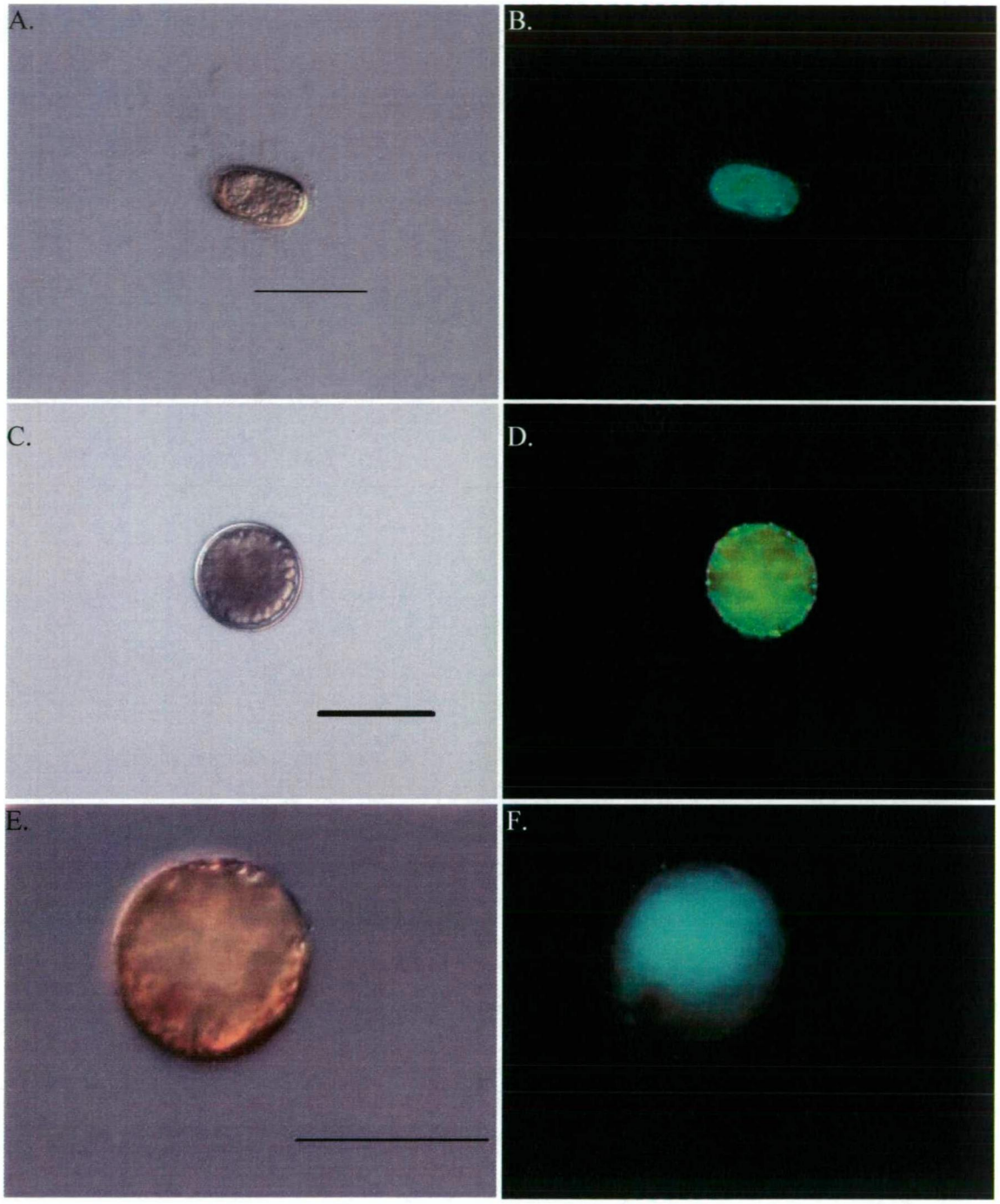

Fig. 2. Fluorescence of treated cysts stained with Sytox® Green showing the ability of the stain to infiltrate the compromised cell walls of dead cysts causing them to fluoresce green in colour when excited with a 450-490 nm light source (450-490 excitation wavelength, 515 emission wavelength). (A) Dead *A. catenella* cyst (20 ppm SeaKleen®); (B) dead *A. catenella* cyst viewed under fluorescent microscope; (C) dead *A. pseudogonyaulax* cyst (600 ppm Peraclean® Ocean); (D) dead *A. pseudogonyaulax* cyst viewed under fluorescent microscope; (E) dead *G. catenatum* cyst (10 ppm SeaKleen®); (F) dead *G. catenatum* cyst viewed under fluorescent microscope. Scale bars=50µm

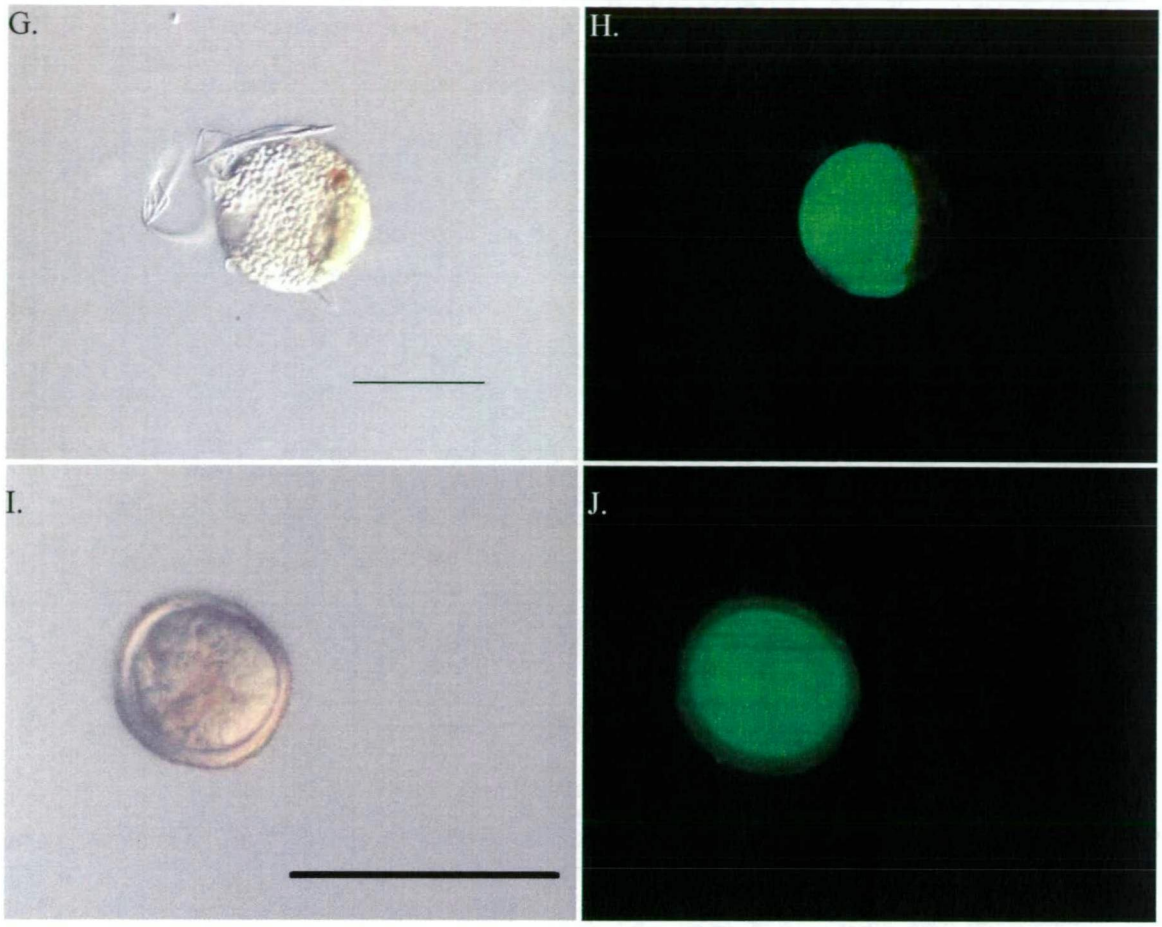

Fig. 2 *cont.*. Fluorescence of treated cysts stained with Sytox® Green showing the ability of the stain to infiltrate the compromised cell walls of dead cysts causing them to fluoresce green in colour when excited with a 450-490 nm light source (450-490 excitation wavelength, 515 emission wavelength). (G) Dead *G. catenatum* cyst (2000 ppm Peraclean® Ocean); (H) dead *G. catenatum* cyst viewed under fluorescent microscope. (I) dead *P. reticulatum* cyst (600 ppm Peraclean® Ocean); (J) dead *P. reticulatum* cyst viewed under fluorescent microscope. Scale bars=50μm

5.5 Discussion

The use of the nucleic acid stain Sytox® Green for the determination of dinoflagellate cyst viability was first demonstrated by Binet and Stauber (2006) using flow-cytometric analysis on heat-treated *Alexandrium catenella* cysts. The present study demonstrated that reliable assessment of dinoflagellate cyst viability can also be achieved by using Sytox® Green in conjunction with fluorescent microscopy. The method was validated against conventional long-term germination experiments. In the present study, the same chemical biocide concentrations were required to inactivate

cysts in both the germination and Sytox® Green viability staining experiments with cysts of *A. catenella, A. pseudogonyaulax, G. catenatum* and *P. reticulatum* killed following 2 weeks exposure to the respective concentrations of 600, 600, 2000 and 600 ppm of Peraclean® Ocean; and 20, 6, 20 and 10 ppm of SeaKleen®. The fluorescent stain infiltrated the compromised cell wall of dead cysts causing them to fluoresce green when exposed to a 450-490 nm light source. Sytox® Green did not stain live cysts. Dead *G. catenatum* cysts did not fluoresce as well as the other test species, except in cases where the cyst wall was completely bleached or removed, nonetheless, live and dead cells were still distinguishable. This may be due to a lower light transmittance through the brown-pigmented sporopollenin cyst wall of *G. catenatum*. At sub-lethal biocide concentrations, total cyst viability was always found to be higher in the Sytox® Green viability staining experiments. This would suggest that either some of the live cysts were stained by Sytox® Green, or alternatively, that the 6-week observation period used in the germination experiments may not have been sufficient for all viable cysts to germinate. Successful germination of cysts clearly necessitates more than just intact cyst wall membranes.

Although the majority of studies on dinoflagellate cysts in ballast water have focused on resistant resting cysts, the hostile ballast tank environment is also likely to promote the formation of immotile temporary cysts, which are also formed when vegetative dinoflagellate cells are exposed to unfavourable conditions such as mechanical shock, nutrient limitation, dark conditions or a rapid change in temperature. Rigby and Hallegraeff (1994) found that some dinoflagellates capable of producing temporary cyst stages survived a 17-day voyage from Japan to Canada. Although temporary resting cysts may only be able to survive in the sediment of ballast tanks for several months (Grzebyk & Berland 1996) as opposed to viable resting cysts that can remain viable in sediments for decades (Hallegraeff and Bolch, 1992; Lewis *et al.*, 1999), temporary cysts are likley to aid dinoflagellates in withstanding the constantly changing conditions inside ballast tanks. Results from the present study indicate that even though temporary cysts lack the robust affinities of resting cysts, they may still require higher biocide concentrations for effective their effective control compared to vegetative cells.

Another interesting result from the present work was that the biocide concentrations required to inactivate *G. catenatum* cysts were considerably higher than previous findings. Gregg and Hallegraeff (2007) inactivated *G. catenatum* cysts following 2 weeks exposure to concentrations of 400 ppm Peraclean® Ocean and 6 ppm of SeaKleen®, whereas in the present work, 2000 ppm of Peraclean® Ocean and 20 ppm of SeaKleen® was required. Possible explanations for this result include loss of toxicity of the chemical biocides or a variation in resistance of different batches of *G. catenatum* cysts. As *A. catenella* and *P. reticulatum* cysts were controlled at similar biocide concentrations in the current work and in the study by Gregg and Hallegraeff (2007), it is likely that the *G. catenatum* cysts used in the present work had an increased resistance to the chemical treatments. This increased resistance may be attributed to the different culture medium used for the production of *G. catenatum* cysts. Gregg and Hallegraeff (2007) used filtered seawater used to produce cysts whereas in the present work cysts were produced in a dilute GSe nutrient medium suggesting that the rise in available nutrients increased cyst resilience possibly by enhancing the physiological properties of the cyst wall or cyst contents. This finding should be taken into consideration when cysts are bulk-produced in culture for the standard assessment of potential ballast water treatments.

In summary, the nucleic acid stain Sytox® Green was capable of infiltrating the compromised cell wall of dead dinoflagellate cysts of *A. catenella, A. pseudogonyaulax, G. catenatum* and *P. reticulatum* causing an increase in green fluorescence when viewed under fluorescent microscopy. The major advantage of the Sytox® Green method is that cyst viability can be determined in several hours compared to the 6 weeks required using the conventional germination method. A rapid technique for determining cyst viability is valuable for not only viability assessments in lab-scale, pilot-scale and shipboard ballast water treatment studies but also for determining cyst viability in ships ballast tanks and port sediments. Apart from assessing dinoflagellate cyst viability, Sytox® Green is a promising tool for viability assessments of other ballast water organisms. For example, to date, it has been successfully used to assess viability in the bacterium *Escherichia coli* (Roth *et al.,* 1997), a wide range of vegetative marine microalgal species (Veldhuis *et al.,* 1997; Veldhuis *et al.,* 2006), the copepod *Calanus helgolandicus* (Buttino *et al.,* 2004), and the nematode worm *Caenorhabditis elegans* (Gill *et al.,* 2003). There are

many practical applications for viability assessments that can be performed quickly. This is particularly true for assessing whether or not a shipboard ballast water treatment system is able to meet the requirements of the International Maritime Organisation's Ballast Water Discharge Standard (Regulation D-2) as this legislation requires viable organisms to be quantified.

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Chapter 6

Review of progress in the development of treatment options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water

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6.1 Abstract

The worldwide transfer and introduction of non-indigenous invasive aquatic organisms via ships' ballast water has been conclusively demonstrated to cause significant ecological, economic and human health impacts. Possible solutions to the problem include; 1) treating ballast water to remove or destroy unwanted organisms; and 2) re-designing the ballast system of new vessels to eliminate the need to transport ballast water. Ballast water exchange is currently the only widely practised interim procedure relied upon to minimise the risk of ballast water invasions but the variable efficacy and operational limitations of this approach have led to significant financial investment in the last two decades in the research and development of more rigorous shipboard ballast water treatment technologies. Specific technologies under consideration include mechanical separation, heat treatment, UV irradiation, ultrasound, de-oxygenation, chemical biocides or active substances and treatment systems that incorporate multiple technologies. To date, no treatment option or multi-component treatment system has proved to be completely effective and approaches trialled have been limited by one or more factors such as cost effectiveness, space and energy requirements, environmental soundness, safety and biological efficacy. Many of these limitations relate to the high flow rates and volumes of water that must be treated, and the presence of sediment in ballast tanks, which reduces the efficacy of many treatment options and provides a habitat for resistant organisms such as resting stages of phytoplankton and zooplankton. Mechanical separation devices would best be used as a primary stage of a treatment system comprising multiple technologies as free-living organisms and sediment below a certain size are likely to be largely unaffected. UV treatment systems are unlikely to eliminate all ballast water organisms, as they are not able to deliver a stable lethal dose across a wide range of water quality conditions and many organisms are resistant to UV exposure or can recuperate after treatment. At the current stage of development, ultrasound treatment would not be considered appropriate for the shipboard treatment of ballast water due to high capital and operating costs and high power requirements. The heating of ballast water using waste heat from ships' engines has been demonstrated to be a practical and cost effective treatment options for eliminating ballast water zooplankton and phytoplankton (including resting stages) but concerns have been expressed that the attainable temperatures may not eliminate bacterial pathogens or

that this approach does not apply to ships traversing colder seas. Promising research has been conducted on several systems that are able to achieve temperatures capable of eliminating bacteria but these technologies are still under development. De-oxygenation by the addition of glucose or reducing agents are not effective ballast water treatment options, however de-oxygenation technologies that are based on the injection of an inert gas are more promising (notably against larval zooplankton) as they are cost effective and do not impact on the aquatic environment as ballast water is re-oxygenated prior to discharge. Biocide dosing systems have low capital costs and power requirements but chemical costs are significant. Chemical treatment costs and space requirements can be significantly reduced by using onboard chemical generators but the capital cost of these systems is significant and all have biological efficacy, safety, operational and environmental (poor biodegradation) concerns. Treatment systems that produce free hydroxyl radicals would be favourable over other chemical treatments as they are claimed to produce less or no toxic by-products at ballast discharge but these technologies have high power requirements. Each treatment option requires further research on their biological and operational efficacy and safety under full-scale shipboard conditions. Accurate sampling and viability testing of phytoplankton and zooplankton size categories in ballast tanks in onboard situations is still problematic. Several promising devices and methods are currently available but effectively eliminating the risk of ballast mediated invasions remains a monumental technological challenge.

Keywords: Ballast water treatment, Invasive species, Bacteria, Phytoplankton, Zooplankton

6.2 Introduction

The requirement for ship ballast water treatment was first formally introduced in 2004 as a component of the Ballast Water Discharge Standard (Regulation D-2) of the International Convention for the Control of Ships Ballast Water and Sediments (IMO, 2004). Once ratified, the Convention requires all ships to conduct ballast water exchange as an interim strategy, and depending on construction date and ballast capacity, ships must use an approved treatment system capable of treating ballast water to strict microbial standards.

Regulation D-2 of the Convention specifies that ships meeting the requirements of the Convention must discharge:

- less than 10 viable organisms per cubic meter greater than or equal to 50 micrometers in minimum dimension, and
- less than 10 viable organisms per millilitre less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension, and
- less than the following concentrations of indicator microbes, as a human health standard:
    - Toxigenic *Vibrio cholerae* (serotypes 01 and 0139) with less than 1 Colony Forming Unit (cfu) per 100 millilitres or less than 1 cfu per 1 gram (wet weight) of zooplankton samples,
    - *Escherichia coli* less than 250 cfu per 100 millilitres, and
    - Intestinal *Enterococci* less than 100 cfu per 100 millilitres.

In response to this, a number of shipboard treatment options are under consideration or under commercial development. Many of these options stem from technologies or procedures currently utilised for industrial and drinking water treatment. The shipboard treatment of ballast water may be achieved during ballasting and/or de-ballasting or when the vessel is *en-route.* Either way this involves retrofitting or modifying the existing ships' infrastructure to accommodate the desired treatment systems. A variety of mechanical/physical and chemical technologies have been

proposed as potential shipboard treatment options. Mechanical treatments include filtration and cyclonic separation and are based on particle-size or specific weight to remove organisms from ballast water (Taylor *et al.,* 2002). These options have the advantage of returning removed organisms directly to the source water and pose no environmental problem. Physical treatment options are also considered environmentally friendly as they focus on changing the physical properties or hydrodynamic characteristics of the water for organism removal (Taylor *et al.,* 2002). It is the chemical treatment options that pose the greatest environmental threat, since large volumes of treated ballast water will be released into ports around the world. Potential chemical treatment options include deoxygenation, pH and salinity adjustment and the use of chemical biocides or the onboard generation of active substances. Currently available and prospective treatment options as of mid 2008 are reviewed and evaluated.

6.3 Mechanical/Physical treatment options

Ballast water exchange

Ballast water exchange (BWE) is currently the only internationally accepted and widely practised procedure relied upon to minimise the risk of ballast water invasions and will become mandatory for existing vessels following the ratification of the IMO Convention until the installation of approved treatment systems is required. Ballast water exchange involves replacing ballast water taken onboard in coastal and port areas, with open oceanic water prior to discharge at subsequent ports of call. Open oceanic water is described by the International Maritime Organisation (IMO) in its International Convention for the Control and management of Ships' Ballast Water and Sediments as water from at least 200 nautical miles from the nearest land and at least 200 metres in depth (IMO, 2004). In theory, BWE acts to reduce the concentration of coastal organisms in the ballast tanks that may be able to invade a recipient port and replaces them with a lower density of oceanic organisms with a low probability of survival in near-shore waters. Ballast water exchange may also be beneficial when a ship is travelling between two freshwater ports, as oceanic water

would cause an increase in salinity to a point where many freshwater organisms may die (Perakis and Yang, 2003).

There exist two methods of conducting BWE: the flow-through method and empty-refill technique. Empty-refill involves discharging the ballast water completely and then refilling the tanks. The flow-through method involves ships continually pumping oceanic water into ballast tanks or holds, either from the bottom or top, allowing them to overflow thus gradually exchanging ballast water. The IMO recommends that the flow-through method be conducted until a water volume of three times the ballast capacity of the ship is pumped through the tanks (IMO, 2004) resulting in approximately a 95% volumetric exchange of the original ballast water (Rigby *et al.*, 1993).

Both ballast water exchange methods likely offer preventive capabilities; however, it is important to realise several shortcomings. Firstly, the ability to safely conduct BWE depends on weather and sea surface conditions making it not always possible to perform. Secondly, it is not 100% effective as most sediment and many bacteria, microalgae and zooplankton species have been shown to remain in the ballast tanks following exchange; and finally, if conducted at the wrong time and location, BWE can result in a greater biological diversity of organisms in the ballast water (Drake *et al.*, 2001; Forbes and Hallegraeff, 2001; Drake *et al.*, 2002; Galil and Hülsmann, 2002; Mimura *et al.*, 2005; Burkholder *et al.*, 2007; McCollin *et al.*, 2007).

Rigby *et al.* (1993) studied the effectiveness of open ocean exchange onboard the bulk carrier MV *Iron Whyalla* and discovered that 37, 13 and 5 % of the original water remained in the ballast tanks after exchanging one, two and three ballast tank volumes respectively. Similar studies have achieved comparable water exchange efficiencies ranging from 93 to 100% (Table 1). However, it must be reinforced that the efficacy of organism removal is a complex issue and is distinct from that of exchange of ballast water. This efficiency will depend on several factors including the nature and behaviour of organisms in the tank, the design and structural configuration of tanks, mixing within the tanks and the type and behaviour of sediments (Rigby and Taylor, 2001; Galil and Hülsmann, 2002).

Table 1. Water exchange efficiency for empty/refill and flow-through ballast water exchange.

Mode of Exchange	% Water Exchanged	Reference
Flow-through, 1 tank volume	63.2	Rigby and Hallegraeff (1993)
Flow-through, 2 tank volumes	86.5	Rigby and Hallegraeff (1993)
Flow-through, 3 tank volumes	95	Rigby and Hallegraeff (1993)
Flow-through, 3 tank volumes	90;99	Taylor and Bruce (2000)
Flow-through	86-96	Villac et al.(2001)
Empty/refill, 1 tank volume	93-100	Wonham et al. (2001)
Flow-through, 3 tank volumes	>99	Taylor et al. (2007)
Flow-through, 2 tank volumes	>99	Taylor et al. (2007)
Empty/refill, 1 tank volume	95	Miller (1998)
Empty/refill, 1 tank volume	95-99	Zhang and Dickman (1999)
Empty/refill, 1 tank volume	95-99	Dickman and Zhang (1999)
Empty/refill, 1 tank volume	86	Locke et al. (1993)

Many studies have found that the organism removal efficiency of BWE does not always correspond to the water exchange efficiency. The majority of studies indicate that the effectiveness of BWE at eliminating phytoplankton and zooplankton in ballast tanks ranges widely from 48-100% (Table 2), however, several researchers have found increases in the diversity and abundance of some taxa (including toxic phytoplankton species) after the exchange process (Macdonald and Davidson, 1998; McCollin *et al.,* 2007a). For example, McCollin *et al.* (2007a) examined the efficacy of ballast water exchange in regional seas using both the empty/refill technique and the flow through method and found that although there was an overall reduction in the abundance of phytoplankton after exchange, this was not consistent between tanks and voyages and in some cases there were increases in the diversity and abundance of harmful diatom and dinoflagellate species. Zhang and Dickman (1999) and Dickman and Zhang (1999) reported a discrepancy in organism removal efficiency by BWE between two different ships. The older vessel was found to have a removal efficiency of only 48% of diatoms and dinoflagellates compared to an 87% reduction in the modern ship after 95-99% replacement of water following ballast exchange using the empty-refill method (Dickman and Zhang, 1999; Zhang and Dickman, 1999). This discrepancy in organism removal efficiency was attributed to the age and design of the ships highlighting the complexity of organism behaviour in ballast tanks.

Table 2. Organism removal efficiency for empty/refill and flow-through ballast water exchange.

Mode of Exchange	% Organism Removal	Reference
Flow-through, 3 tank volumes	75 (original phytoplankton)	Rigby and Hallegraeff (1993)
Flow-through, 3 tank volumes	90-100 (selected taxa)	Taylor and Bruce (2000)
Flow-through	86-96 (phytoplankton)	Villac *et al.* (2001)
Empty/refill, 1 tank volume	80-100 (coastal organisms)	Wonham *et al.* (2001)
Flow-through, 3 tank volumes	90-100 (original phyto- and zooplankton taxa)	Taylor *et al.* (2007)
Flow-through, 2 tank volumes	90-100 (original phyto- and zooplankton taxa)	Taylor *et al.* (2007)
Empty/refill, 1 tank volume	87 (diatoms and dinoflagellates)	Zhang and Dickman (1999)
Empty/refill, 1 tank volume	48 (diatoms and dinoflagellates)	Dickman and Zhang (1999)
Flow-through	<90 (coastal organisms)	Ruiz and Hines (1997)
Empty/refill, 3 tank volumes	>95 (coastal organisms)	Ruiz and Hines (1997)
Empty/refill, 1 tank volume	67 (plankton)	Locke *et al.* (1993)

Studies assessing the effects of BWE on the microbial ecology of ships ballast water found no significant difference in the microbial abundance and biomass between exchanged and original ballast water (Drake *et al.*, 2002; Mimura *et al.*, 2005). Nonetheless, BWE remains a valuable interim option and improvements to ships' designs may increase the efficiency of BWE. The operating cost of BWE is approximately US$0.01-0.02 per tonnes of ballast water but higher costs are involved if a ship requires additional equipment for safe or effective exchange (Taylor *et al.*, 2002).

Filtration

Filtration is an environmentally sound technique for the control of ballast water organisms that works by capturing organisms and particles as water passes through a porous screen, filtration medium or stacks of special grooved disks. It has been proposed as a single treatment option and as the primary treatment component of a system that uses a combination of technologies (Muntisov *et al.*, 1993; Parsons and Harkins, 2000, 2002; Tang *et al.*, 2006; Cangelosi *et al.*, 2007). Filtration systems under consideration for ballast water treatment include self-cleaning backwashing screen filters, disk filters and crumb rubber filtration. Potential filters must handle

high flow rates and pressure, together with sediment and other objects present in the water.

Fifty micron filtration is likely to be effective in removing most zooplankton and significant amounts of microalgae from ballast water, while 20 μm filtration is necessary to remove most dinoflagellate cysts. Membrane filters are needed for the removal of most protozoans, bacteria and viruses. A membrane filter pore size of 0.20 μm is needed for producing bacteria-free filtrates (Gardner and Peel, 1991). High capital and operating costs together with the large volumes and high flow rates of water involved in ballasting exclude the use of membrane filters in ballast water treatment. Membrane filtration has been suggested a valid option in circumstances where cost is not a major issue. For example, membrane filtration plus chlorination has been proposed for the treatment of ballast water on vessel types with small amounts of ballast, such as cruise ships (Oemcke, 1999).

Several studies have shown self-cleaning backwashing filtration systems to be effective in the removal of significant amounts of phytoplankton, zooplankton and particulate matter from water bodies (Table 3). Parsons and Harkins (2000, 2002) examined the efficacy of automatic backwash screen filtration with mesh sizes of 25, 40, 50 and 100 μm and an automatic backwash disk filter with a 100 μm rating. Full-scale trials demonstrated that the 25, 40 and 50 μm filter screens and 100 μm disk filter removed similar quantities of organisms and particles with an overall count efficiency (based upon the total count of all particles above the nominal filter rating) of 88, 88.7, 91.9 and 91.4% respectively. The results for the 100 μm screen filter were somewhat lower with an average overall count efficiency of 61.8%.

Biological testing has shown that 25 and 50 μm screen filters can significantly change the structure of the planktonic community (Cangelosi *et al.*, 2007). Twenty five micron filtration is significantly more effective than 50 μm at removing zooplankton and microalgae. The 25 μm filter removed almost >99% of macrozooplankton, 99% of dinoflagellates and 81% of microzooplankton and small phytoplankton, whereas the 50 μm filter removed just under 97% of the macrozooplankton, 91% of dinoflagellates and just over 70% of microzooplankton and small algal species

(Cangelosi *et al.*, 2007). Although 25 μm filtration can remove more organisms from the water, it is much less operationally efficient. On a percentage basis, 25 μm filtration exhibited a 60% greater loss of flow than the 50 μm filter screen due to self-cleaning backwash operations (Parsons and Harkins, 2000). Disk filters attained the highest net flow rate between 93.3 and 96.1 % (Parsons and Harkins, 2002).

Bacteria associated with plankton and other suspended matter are also reduced by 25 and 50 μm filters, however, free-living bacteria or bacteria associated with organisms less than 25μm are unaffected (Cangelosi *et al.*, 2001). Huq *et al.* (1996) found significant removal of *Vibrio cholerae* was achieved by filtration of their intermediate zooplankton host.

Design improvements may increase the performance of 25 μm filter screens, however the results to date suggest that current 25 μm screen filters are not suitable for shipboard applications. Parsons and Harkins (2000) recommend the 50 μm screen filter for use on ships, however Parsons and Harkins (2002) indicate that disk filters are more attractive than screen filters when evaluated from the perspectives of particle removal efficiency, backwash frequency and net ballast flow rate, maintainability and system cost. These systems are currently operating at flow rates of 340 m^3/h, which is well below the ballasting rates of a typical bulk carrier (up to 5,000 m^3/h) (National Research Council, 1996). Additionally, filtration is relatively expensive costing an estimated US$0.06-0.19 per tonne of ballast water (including capital cost) (Taylor *et al.*, 2002; Perakis and Yang, 2003).

Table 3. Removal efficiency of potential ballast water filtration options.

Filter Type	Filter Screen/nominal Pore Size (μm)	Removal Efficacy (%)	Net Lost Flow Due to Backwash (%)	Reference
Screen Filters	25	94-100 (macrozooplankton)	10.6-21.2	Cangelosi *et al.* (2007)
		81-85 (microzooplankton)		Cangelosi *et al.* (2007)
		85-91 (total zooplankton)		Cangelosi *et al.* (2007)
		99 (dinoflagellates)		Cangelosi *et al.* (2007)
		37.5 (total phytoplankton)		Cangelosi *et al.* (2007)
		88 (particles 25 μm or greater)		Parsons and Harkins (2000)
	40	88.7 (particles 40 μm or greater)	N/A	Parsons and Harkins (2002)
	50	94-97 (macrozooplankton)	6.8-13.5	Cangelosi *et al.* (2007)
		71-81 (microzooplankton)		Cangelosi *et al.* (2007)
		78-89 (total zooplankton)		Cangelosi *et al.* (2007)
		91 (dinoflagellates)		Cangelosi *et al.* (2007)
		8.3 (total phytoplankton)		Cangelosi *et al.* (2007)
		50-70 (particles greater than 50 μm)		Anderson (2007)
		60-95 (bivalve and gastropod larvae)		Waite *et al.* (2003)
		91.9 (particles 50 μm or greater)		Parsons and Harkins (2000)
		ineffective against *Phaeocystis globosa* (4-6 μm cell diameter; 20-500 μm colony diameter)		Veldhuis *et al.* (2006)
		ineffective (particles >2-<63 μm)		Veldhuis *et al.* (2006)
		86 (zooplankton)		Veldhuis *et al.* (2006)
	100	83 (macrozooplankton)	N/A	Cangelosi *et al.* (2007)
		68 (microzooplankton)		Cangelosi *et al.* (2007)
		79 (total zooplankton)		Cangelosi *et al.* (2007)
		61.8 (particles 100 μm or greater)		Parsons and Harkins (2000)
Disk Filters	55	80 (organisms >50 μm)	N/A	Wright *et al.* (2007c)
	100	91.4 (particles 100 μm or greater)	4.9-6.8	Parsons and Harkins (2002)
Crumb Rubber	≥500 (media size)	86.8 (particles 10 μm or greater)	N/A	Xie and Chen (2004)
		93.6 (particles 15 μm or greater)		Xie and Chen (2004)
		51.7 (particles 2 μm or greater)		Xie and Chen (2004)
		70 (phytoplankton)		Tang *et al.* (2006)
		45 (zooplankton)		Tang et al. (2006)

To date, screen or disk filters have been the favourable filter type for ballast water treatment; however, recently there has also been some interest in the use of crumb rubber, a scrap tyre-derived material, as a filtration medium. Preliminary laboratory experiments conducted at low flow rates indicated that crumb rubber filtration was capable of effectively removing small particles (>2 μm), however further experimentation showed effectiveness of this system was found to be considerably lower than screen and disk filters achieving maximum organism removal efficiencies of 70% for phytoplankton and 45% for zooplankton and was only operational at low flow rates (24.4-73.4 m^3/h) (Table 3).

Clearly, filtration alone cannot prevent the transfer of all ballast water organisms, as free-living organisms below a certain size (25 or 50 μm) are likely to be largely unaffected. This would include most bacteria and viruses, many diatoms, dinoflagellates, other phytoplankton species, microzooplankton and various resting stages. Bacteria associated with crustaceans may be reduced, but filtration acts to primarily remove larger aquatic organisms (>50 μm) and reduce the sediment load during ballasting. Improvements in filter technology and design may allow the use of smaller pore sizes; however at present 50 μm filtration is operationally viable. Filtration therefore would best be used as a primary treatment stage of a treatment system comprising multiple technologies. These technologies may include heat, UV, ultrasound treatment or dosing with chemical biocides or active substances.

Cyclonic separation

Cyclonic separators or hydrocyclones are simple mechanical devices that operate by centrifugal action causing heavier particles to move to the outside where they are captured by a weir-like feature near the discharge point (Parsons and Harkins, 2002). These devices have the advantage of requiring minimal maintenance due to having virtually no moving parts, and pose no environmental risk as collected particles and organisms are returned directly to the source water.

Several studies have evaluated the efficiency of cyclonic separation in removing organisms and sediment from water (Table 4). In most cases, performance was found

to be sub-optimal and significantly inferior to screen and disk filters. Jelmert (1999) tested removal efficiency using dense cultures of the dinoflagellate *Prorocentrum minimum* (10-15 μm), the green alga *Tetraselmis* (10-15 μm) and two isolates of marine bacteria (0.2-1 μm) as model organisms. No significant removal of either *Prorocentrum minimum* or *Tetraselmis sp.* was found with removal efficiencies varying between 10 and 30%. As could be expected, no separation effect on bacteria was observed. Parsons and Harkins (2002) found similar removal efficiencies. Parsons and Harkins (2002) tested a hydrocyclone on a barge installation suggested to be effective in removing particles above the 50-100 μm range. The device removed very little material below about 400 μm resulting in an overall removal efficiency of only 33% of particles above 100 μm. Veldhuis *et al.* (2006) highlights one advantage of hydrocyclones; although the device was not very effective in reducing total suspended solids, it was capable of altering gelatinous phytoplankton colonies in a manner which greatly reduced the clogging of the secondary self-cleaning filter.

Table 4. Organism and sediment removal efficacy of cyclonic separators

Manufacturer	Flow Rate	Removal Efficiency (%)	Net Flow Loss Due to Discharge Path	Reference
OptiMarin	N/A	15-40 (of particles <50 μm)		Hesse *et al.* (2004)
Velox Technology Inc.	68-79 m^3/h	10-30 (*Prorocentum minimum* and *Tetraselmis* sp.)	10%	Jelmert (1999)
	68-79 m^3/h	13.7 (*Artemia* sp. cysts)		Jelmert (1999)
	68-79 m^3/h	8.3 (*Artemia* sp. nauplii)		Jelmert (1999)
	312-350 m^3/h	ineffective (phytoplankton)	10%	Sutherland *et al.* (2001)
Greenship Ltd	N/A	100 (particles ≥20 μm)		MEPC (2007a)
	N/A	80 (particles ≥10 μm)		MEPC (2007a)
Hamann AG	N/A	>90 (particles >40 μm)		Hamann AG (2007)
	530 m^3/h	ineffective (*Phaeocystis globosa*, particles >2-<63 μm)		Veldhuis *et al.* (2006)
Krebs Engineers	342 m^3/h	<15 (zooplankton)		Waite *et al.* (2003)
	342 m^3/h	no effect (phytoplankton)		Waite *et al.* (2003)
HydeOptiMarin	340 m^3/h	33 (particles ≥100 μm)		Parsons and Harkins (2002)

The above results suggest that the cyclonic separation of small organisms, and organisms with a specific gravity close to that of water, is minimal. This would include many organisms such as viruses, protozoans, bacteria, phytoplankton, chaetognaths and jellyfish. Oemcke (1999) suggests that dinoflagellate cysts should

be removable from ballast water using cyclonic separation as they have a specific gravity of greater than 1.05 and typically greater than 1.1. However, given the performance of the systems tested to date, this would prove questionable. The estimated cost of cyclonic separation is US$0.05-0.26 per tonne of water (including capital cost) (Taylor *et al.,* 2002; Perakis and Yang, 2003), which is comparable to filtration. Cyclonic separation does have the advantage of being able to operate continuously under high flow rates (~3000 m^3/h) and may prove an effective primary treatment option as it acts to improve water clarity prior to secondary treatment (Sutherland *et al.,* 2001). It can also remove large, dense particles thus protecting secondary treatment devices, but current systems are unlikely to remove a significant amount of zooplankton, microalgae and bacteria from water. The particle removal efficiency of some newer designs is suggested to be much greater. For example, Greenship Ltd of the Netherlands has developed a hydrocyclone device that is claimed to be capable of removing 100% of particles 20 μm and larger, and 80% of the particles greater than 10 μm (MEPC, 2007a).

Heat treatment

The use of heat for killing ballast water organisms has received considerable attention as it is an environmentally attractive option, is potentially cost effective and has been shown to eliminate a wide range of aquatic organisms (Table 5). Several different heat treatment processes are suggested as potential shipboard ballast water treatment options (Table 6). One proposed method uses waste heat from the ships engine cooling system and exhaust to treat ballast water (Hallegraeff *et al.,* 1997; Rigby *et al.,* 2004). Results from a shipboard trial on the MV *Iron Whyalla* suggest that heating ballast water to 38-45°C is achievable and may be an effective way to kill zooplankton and phytoplankton organisms in ballast tanks (Rigby and Hallegraeff, 1993; Rigby *et al.,* 1998; Rigby *et al.,* 1999). Laboratory experiments have demonstrated: 1) that phytoplankton and dinoflagellate cysts are readily killed when exposed to temperatures in this range, and 2) inactivation time decreases as the temperature is increased (Hallegraeff *et al.,* 1997; Forbes and Hallegraeff 2001). Most vegetative microalgal cells are readily killed at temperatures as low as 35°C with exposure times between 30 min and 5 h. Some resistant species survive such

treatments requiring higher temperatures and/or longer exposure times for mortality to occur. The inactivation of the green alga *Dunaliella tertiolecta* and *Nannochloropsis oculata* required temperatures around 42.5°C at longer exposure times of 24 h (Hallegraeff *et al.*, 1997). Dinoflagellate cysts are more resistant to heat treatment than vegetative cells, however can still be killed by temperatures that are considered attainable inside ballast tanks. *Gymnodinium catenatum* cysts survived a 1 h treatment at 35°C with effective inactivation achieved at exposures ranging from 1 h at 37.5°C, to 2 min at 38-40°C, and 30 s at temperatures of 44.5-46.3°C (Bolch and Hallegraeff, 1993; Hallegraeff *et al.*, 1997). Similarly, *Alexandrium* cysts have been inactivated in 4.5 h at 38°C and 3 min at 45°C (Montani *et al.*, 1995; Hallegraeff *et al.*, 1997). The heating of ballast water to around 40°C also has the advantage of being able to effectively treat organisms present in ballast sediment (Stocks *et al.*, 2004a), with the exception of bacteria. Rigby and Taylor (2001) indicated that higher temperatures are experienced at the bottom of tanks as in their system the heated ballast water was pumped in from the bottom.

However, these low temperature strategies (<45°C) will be largely ineffective for controlling most pathogens and concern have been expressed that mild heating of ships' ballast water may stimulate the growth of pathogenic bacteria such as *Vibrio cholerae*. Desmarchelier and Wong (1998) dispelled such concerns on the basis of *V. cholerae* being severely nutrient limited in typical ballast water conditions. Vegetative bacteria, fungi and viruses are generally killed at temperatures in the range of 60-100°C and temperatures exceeding 100°C are usually required to kill bacterial spores, although the level of heat resistance varies widely according to the species (Gardner and Peel, 1991). Marine and estuarine bacteria, including *E. coli* and *V. cholerae*, require significantly lower heat treatments of 55-75°C for complete inactivation as compared to enterobacteria associated with warm-blooded animals (Gardner and Peel, 1991; Rigby *et al.*, 2004)

Several studies have tested systems that use additional heat exchangers capable of achieving temperatures of 55-80°C for short periods (Mesbahi *et al.*, 2007a; Quilez-Badia *et al.*, 2008). These short exposure high temperature treatments achieved a reduction in zooplankton of up to 95% and significant reductions of up to 90% for

phytoplankton. Most importantly, these treatment systems were capable of causing a 95% reduction of bacteria but there was no significant increase in mortality when the treatment temperature was increased from 55 to 80°C (Mesbahi *et al.,* 2007a; Quilez-Badia *et al.,* 2008). One explanation for the 95% reduction in bacteria was that the vegetative bacterial cells were killed at the lowest treatment temperature (55°C) while the surviving 5% consisted of bacterial spores. As none of the bacterial indicator species listed in the IMO discharge standard are spore forming species it is suggested that they should be relatively easy to kill using heat (Quilez-Badia *et al.,* 2008). Estimated capital costs of this high temperature treatment system are between US$350,000 and 400,000 for ballast flow rates of 1,000 to 3,500 m^3/h and estimated operational costs (based on increased fuel consumption) range from over US$100 for 1,000 m^3/h to over US$600 for 3,500 m^3/h (Mesbahi *et al.,* 2007b).

These results indicate that heat treatment using waste heat from ship engines together with additional heat exchangers deserves further investigation as a stand-alone treatment for ballast water as the temperatures able to be reached in inboard situations have been demonstrated to kill zooplankton, phytoplankton (including resting stages) and vegetative marine bacteria. Additionally, the treatment option is considered to be cost effective (US$0.05-0.17 per tonne excluding capital costs) but some concerns exist over the time and energy required to heat ballast water to the necessary temperature (notably for ships operating in colder seas) and possible environmental impacts following the discharge of large volumes of heated water. It is suggested that heated water should be cooled to less than 10°C above the ambient temperature prior to discharge (ICES WGBOSV, 2006).

Recently, the use of a microwave heating system has been proposed as a ballast water treatment option. Initial laboratory experiments have shown that this system is capable of achieving a maximum temperature of ~89°C very rapidly (100-200 seconds) and when combined with an additional heat exchanger, temperatures in excess of 100°C can be reached (Boldor *et al.,* 2008a,b). Biological efficacy tests showed that *Artemia* adults, *Artemia* nauplii, *Crassostrea virginica* larvae and the microalgae *Nannochloropsis oculata* are completely eliminated at temperatures of 43, 47, 51 and 53°C, respectively, within several minutes using the microwave heating

system (Boldor *et al.,* 2008a). Slightly higher temperatures of 64°C were required for the complete inactivation of *Artemia* cysts (Boldor *et al.,* 2008b). No data are available on bacterial efficacy but it would be expected that marine bacteria would be killed by the temperatures attainable by this device. This technology has higher heating rates compared to conventional heating methods but current systems are only operating at flow rates of 1-2 L/min. The major factors that would negate the use of technology for the shipboard treatment of ballast water are the high energy consumption of the system and high cost. Current cost estimates are about US$2.55 per tonne of ballast water for a system without a heat exchanger and $US1.09 for a system that has a heat exchanger (Boldor *et al.,* 2008a). Nevertheless, this is a promising technology that is still in its infancy and if costs and energy requirements can be significantly reduced, it may provide an effective treatment option.

Table 5. Summary of temperatures required for the complete inactivation or mortality of aquatic organisms.

Organism Group	Species	Treatment	Reference
Marine bacteria			
	Marine bacteria including *Vibrio cholerae* and *Escherichia coli*	55-75°C	Rigby *et al.* (2004); Gardner and Peel (1991)
	Vibrio cholerae	73°C, 30 sec	Thornton and Chapman (2004)
	Vibrio cholerae	65°C, 2 min	Thornton and Chapman (2004)
Microalgae			
Diatoms	*Detonula pumila*	35°C, 1 h	Forbes and Hallegraeff (2001)
	Pseudo-nitzschia cuspidata	35°C, 1 h	Forbes and Hallegraeff (2001)
	Skeletonema costatum	35°C, 1 h	Forbes and Hallegraeff (2001)
	Thalassiosira rotula	35°C, 1 h	Forbes and Hallegraeff (2001)
	Amphora sp.	35°C, 5 h	Forbes and Hallegraeff (2001)
	Navicula sp.	35°C, 5 h	Forbes and Hallegraeff (2001)
	Navicula jeffreyi	35°C, 5 h	Forbes and Hallegraeff (2001)
Chlorophyte	*Dunaliella tertiolecta*	42.5°C, 24 h	Hallegraeff *et al.* (1997)
Raphidophyte	*Chattonella* sp. cysts	45°C, 3 min	Montani *et al.* (1995)
Picoplankton	*Nannochloropsis oculata*	53°C, 100 sec	Boldor *et al.* (2008a)
	Nannochloropsis oculata	42.5°C, 24 h	Hallegraeff *et al.* (1997)
Dinoflagellates	*Gymnodinium catenatum*	35°C, 30 min	Hallegraeff *et al.* (1997)
	Gymnodinium catenatum cysts	38-40°C, 2 min	Bolch and Hallegraeff (1993)
		44.5-46.3°C, 30 sec	Bolch and Hallegraeff (1993)
		37.5°C, 1 h	Bolch and Hallegraeff (1993)
	Alexandrium catenella cysts	42°C, 30 min	Hallegraeff *et al.* (1997)
		40°C, 75 min	Hallegraeff *et al.* (1997)
		38°C, 4.5 h	Hallegraeff *et al.* (1997)
	Alexandrium sp. cysts	45°C, 3 min	Montani *et al.* (1995)
	Scrippsiella sp. cysts	45°C, 3 min	Montani *et al.* (1995)
	Gymnodinium sp. cysts	45°C, 3 min	Montani *et al.* (1995)
	Protoperidinium sp. cysts	45°C, 3 min	Montani *et al.* (1995)
	Gyrodinium sp. cysts	45°C, 3 min	Montani *et al.* (1995)
Macroalgae			
	Undaria pinnatifida spores	35-40°C, 0.9-42 min	Mountfort *et al.* (1999b)
	Undaria pinnatifida gametophyte	55°C, 6 sec	Forrest and Blakemore (2006)
Zooplankton			
Molluscs	*Dreissena polymorpha*	36°C, 10 min	Jenner and Janssen-Mommen (1992)
	Crassostrea gigas larvae	40-48°C, 6-97 min	Mountfort *et al.* (1999b)
	Crassostrea virginica	48.5°C	Sellers and Stanley (1989)
	Corbicula fluminea	44°C	Graney *et al.* (1983)
	Crassostrea gigas spat	39-45°C, 2-167 min	Rajogopal *et al.* (2005)
	Brachidontes striatulus	43°C, 135 min	Gunasingh Masilamoni *et al.* (2002)
	Crassostrea virginica	51°C, 100 sec	Boldor *et al.* (2008a)
	Mytilus edulis	50-60°C, 30-60 sec	Park *et al.* (1998)
	Balanus sp.	55-60°C, 10-15 sec	Park *et al.* (1998)
Echinoderms	*Coscinasterias calamaria* larvae	39-44°C, 1-35 min	Mountfort *et al.* (1999b)
	Arachnoides placenta	37°C, 48 h	Chen and Chen (1992)
Crustaceans	*Acartia tonsa*	30-35°C, 48 h	Heinle (1969)
	Artemia salina nauplii	43°C, 100 sec	Boldor *et al.* (2008a)
	Artemia salina adults	47°C, 100 sec	Boldor *et al.* (2008a)
	Artemia salina cysts	64°C, 100 sec	Boldor et al. (2008b)

Table 6. Comparison of achievable temperature, biological efficacy and estimated cost of different potential shipboard ballast water heat treatment options

Treatment Process	Attainable Temperature	Biological Efficacy	Estimated Cost (US$ per tonne)	Reference
Engine waste heat	35-38°C after 24-30 h	100% of zooplankton and phytoplankton	0.056 (including capital costs)	Rigby and Hallegraeff (1993)
	37-38.4°C after 24-30 h	100% of zooplankton and most phytoplankton		Rigby *et al.* (1998); Rigby et al. (2004)
Heat exchangers	55-80°C for 1-2 sec	95% of zooplankton 63-90% of phytoplankton 95% of bacteria	0.10-0.17 (excluding capital costs)	Quilez-Badia *et al.* (2008); Mesbahi *et al.* (2007b)
Microwave heating	69-89°C in 100-200 sec	100% of *Artemia salina* adults, *Artemia salina* nauplii, *Crassostrea virginica* larvae and *Nannochloropsis aculata*	2.55 (including capital costs)	Boldor *et al.* (2008a)
Microwave heating and additional heat exchanger	73->100°C in several mins	100% of *Artemia salina* cysts	1.09 (including capital costs)	Boldor *et al.* (2008a,b)

Radiation

The biocidal action of electromagnetic radiation has been known for many years. Three wavelength bands are of interest for the control of organisms: gamma rays, microwaves and ultra-violet rays. The use of gamma and microwave radiation has been suggested as a possible ballast water and residue treatment (Muntisov *et al.*, 1993). However, with current technology, the high energy requirements together with high capital and operational costs are likely to prohibit these technologies from shipboard ballast water treatment. Ultra-violet irradiation, on the contrary, is considered economically viable and has been demonstrated as a feasible ballast water treatment option.

Ultra-violet (UV) light inactivates organisms by causing photochemical alterations of cell material and has been demonstrated effective against a variety of microorganisms (Chang *et al.*, 1985). UV disinfection is relatively insensitive to temperature changes (Severin *et al.*, 1983) and effectiveness varies with microbe type. Effectiveness depends largely upon the size and morphology of the organism. For example, microalgae require higher inactivation dosages than bacteria and viruses due to their

larger size and pigmentation (Rigby and Taylor, 2001). However, care must be taken when deciding lethal UV doses as phytoplankton and bacteria exhibit recovery processes known as photoreactivation and dark repair. Table 7 summarises the effectiveness of UV irradiation against aquatic organisms.

Table 7. Effectiveness of UV irradiation against aquatic organisms

Organism	UV Dosage	Efficacy (%)	Reference
Bacteria and protozoa			
Cryptosporidium parvum oocysts	230 mW-s/cm^2	99	Morita *et al.* (2002)
Cryptosporidium	330 mW-s/cm^2	99	Buchholz (1998)
Escherichia coli	7-16 mW-s/cm^2	99	Buchholz (1998)
Escherichia coli	25-60 mW-s/cm^2	>99	Waite *et al.* (2003)
Marine bacteria	96-115 mW-s/cm^2	98.8-99.6	Jelmert (1999)
Marine bacteria	200 mW-s/cm^2	25-90	Cangelosi *et al.* (2001)
Staphylococcus aureus	7 mW-s/cm^2	99	Buchholz (1998)
Total coliform bacteria	25-60 mW-s/cm^2	>99	Waite *et al.* (2003)
Vibrio cholerae	7-13 mW-s/cm^2	99	Buchholz (1998)
Vibrio salmonicida	2.7 mW-s/cm^2	99.999	Liltved *et al.* (1995)
Vibrio anguillarum	2.7 mW-s/cm^2	99.999	Liltved *et al.* (1995)
Vibrio anguillarum	22 mW-s/cm^2	99.99	Sugita *et al.* (1992a)
Yersinia ruckeri	2.7 mW-s/cm^2	99.999	Liltved *et al.* (1995)
Cyanobacteria			
Blue-green algae	300-600 mW-s/cm^2	90	Buchholz (1998)
Microalgae			
Amphidinium sp., *Gymnodinium catenatum*	<50 mW-s/cm^2	100	Oemcke (1999)
Skeletonema costatum	2.5 KW total output	100	Sutherland *et al.* (2001)
Tetraselmis sp.	96-115 mW-s/cm^2	87.6	Jelmert (1999)
Prorocentrum minimum	96-115 mW-s/cm^2	84.7	Jelmert (1999)
Gymnodinium catenatum cysts	1,600 mW-s/cm^2	ineffective	Oemcke (1999)
Chattonella sp. cysts	30 lux	94	Montani *et al.* (1995)
Scrippsiella sp. cysts	30 lux	52	Montani *et al.* (1995)
phytoplankon	N/A	78	Mesbahi (2004)
Zooplankton			
Artemia sp. nauplii	96-115 mW-s/cm^2	99.5	Jelmert (1999)
Artemia sp. cysts	96-115 mW-s/cm^2	26	Jelmert (1999)
Live organisms	200 mW-s/cm^2	88.7	Wright *et al.* (2007c)
Nematode eggs	92 mW-s/cm^2	99	Buchholz (1998)
Total zooplankton	N/A	65	Mesbahi (2004)
Total zooplankton	200 mW-s/cm^2	>95	Wright *et al.* (2004)

Heterotrophic bacteria are readily inactivated by UV irradiation. Laboratory experiments conducted by Liltved *et al.* (1995) discovered a dosage rate of 2.7 mW-s/cm^2 resulted in more than 5-log (99.999%) reduction of three fish pathogenic bacteria, *Vibrio salmonicida, Vibrio anguillarum* and *Yersinia ruckeri*, whilst Sugita

et al. (1992a) found a higher dosage rate of 22 mW-s/cm^2 was required to inactivate *Vibrio anguillarum*. This difference in reported UV sensitivity of *Vibrio anguillarum* may possibly be explained by variations in experimental factors such as temperature and water clarity, or different bacterial isolates may respond differently to UV irradiation.

Dinoflagellate cysts have been documented to be resistant to UV irradiation. Oemcke (1999) demonstrated that UV light can effectively control the dinoflagellate *Amphidinium* sp. and vegetative cells of *Gymnodinium catenatum* at a dosage rate of <50 mW-s/cm^2; however cysts of *G. catenatum* were not destroyed at dosage rates of up to 1,600 mW-s/cm^2. Similarly, Montani *et al.* (1995) reported that UV irradiation is not an effective treatment for the destruction of microalgal cysts as germination of all experimental species was still evident after 2 h exposure.

Several UV treatment systems have reached the shipboard and pilot-scale stage. These systems are comprised of UV-C lamps arranged in circular formations and can treat ballast water during intake and discharge. Jelmert (1999) examined the effectiveness of UV irradiation on *Artemia* nauplii, *Artemia* cysts, the dinoflagellate *Prorocentrum minimum*, the green alga *Tetraselmis* sp. and two isolates of marine bacteria following a primary cyclonic separation treatment. UV dosage rates ranging from 96-115 mW-s/cm^2 resulted in 84.7 and 87.6% mortality in the two algal species and a 2.3 and 1.9 log reduction in the marine bacteria. A mortality of 95% was achieved for *Artemia* nauplii and the germination of *Artemia* cysts was reduced by 26% (Jelmert, 1999). This system appears inferior to the filtration and UV set-up analysed by Cangelosi *et al.* (2001), whereby dinoflagellates were reduced in concentration by >95%, highlighting how poor water clarity reduces the effectiveness of UV treatment. The low removal efficiency of particulate matter in the cyclonic separator caused a reduction in UV transmittance resulting in a lower kill rate in the secondary UV treatment. A UV transmittance of 30-45% resulted in a mean inactivation of 25% for bacteria, while a transmittance of >90% resulted in 90% inactivation (Cangelosi *et al.*, 2001) suggesting that bacteria are shielded from the UV treatment in the presence of high sediment loads, thus requiring a physical pre-treatment process such as filtration for effective removal. Conversely, Waite *et al.* (2003) examined the effectiveness of a pilot-scale treatment system consisting of a

hydrocyclone, 50 μm self-cleaning screen filter and a UV system and found that pre-treatment via screens or hydrocyclones is not required to enhance the removal efficiency of *E. coli* with UV treatment. Results also indicated that the dosage rate delivered by the UV system (25-60 mW-s/cm^2) was not efficient or successful at killing phytoplankton in ballast water and the bactericidal effect was short-lived, as regrowth occurred after 18 h.

Onboard testing has been carried out on the USS *Cape May* using a UV disinfection system rated at 200 mW-s/cm^2. Efficacy tests showed that when UV transmittance was in excess of 90%, zooplankton mortalities greater than 95% occurred, phytoplankton growth was reduced but the levels of bacteria were greatly increased (due to the decomposing organic matter) (Wright *et al.,* 2004). One of the latest treatment systems based on UV irradiation has been installed on the cruise ship *Coral Princess* (Wright *et al.,* 2007c). This treatment system consisted of a 50 μm disk filter and a medium-pressure UV disinfection unit also rated at 200 mW-s/cm^2. Efficacy testing of the system revealed an overall reduction in live organisms of 69.7-99.1% (mean 87%) but it did not conform to the IMO's D-2 discharge standard as >100 individuals larger than 50 μm per tonne of ballast water survived the treatment. The UV system was 100% effective against coliform bacteria however no difference was found in colony counts of heterotrophic bacteria in treated water relative to untreated controls.

Acher *et al.* (1997) propose a different UV system that concentrates UV irradiation on a transparent quartz pipe through which water or effluent is passed. It was suggested that this system is superior to existing shipboard installations as it can operate in waters high in sediment. Preliminary experiments conducted in turbid waters obtained promising results with poliovirus and several other human pathogens from infested water controlled with dosage rates between 10 and 33 mW-s/cm^2 (Acher *et al.,* 1997). However, currently this system can only operate with small water volumes at a low flow rate excluding it as a ballast water treatment option.

In summary, the current UV systems would be unlikely to eliminate all ballast water organisms as they seem unable to deliver a stable lethal dose to the entire ballast flow

across a range of water quality conditions. The presence of sediment is likely to drastically reduce the efficacy of UV as small organisms such as bacteria would be shielded from the treatment. Additionally, as many organisms, including dinoflagellate cysts, are resistant to UV irradiation, fine filtration would be necessary for their removal. A primary treatment step may also function to protect the UV source, improve water clarity, thus increasing UV transmittance, and reduce power requirements and maintenance costs. UV treatment systems would best be suited on ships with moderate ballast flow rates (1000 m^3/h) because once flow rates exceed this rate, these types of systems would require a prohibitively large amount of power and could be restricted by space limitations. The estimated capital cost for a UV ballast water treatment system ranges from US$300,000 to $400,000 depending on the manufacturer with operational costs of approximately US$0.065-0.26 per tonne of ballast water (Perakis and Yang, 2003; Sassi *et al.,* 2005; Lloyd's Register, 2007). Although UV is considered an environmentally sound treatment option, possible environmental concerns include the release of ballast water containing genetically mutated organisms, i.e. organisms that manage to survive the UV treatment but have damaged DNA, and propulsion vibrations from ships engines have caused the UV lamps to rupture releasing mercury (Swanson and Perlich, 2006).

Ultrasound treatment

The destruction of microorganisms by acoustic technologies has been of considerable interest for over 80 years. The mechanical effects of ultrasound on biological systems in a liquid medium are mainly thought to be due to cavitation although pressure wave deflections and possibly the degassing effect of ultrasound may also contribute to the mortality of marine organisms (Mason *et al.,* 2003; Rigby and Taylor, 2001).

Ultrasound treatment is a relatively new technology for the treatment of ships' ballast water. Data concerning the efficacy of ultrasonic treatment systems in ballast water situations indicate that zooplankton may be effectively controlled, yet the effectiveness against microalgae and bacteria has not been proven. Holm *et al.* (2008) showed that the survival of zooplankton larger than 100 μm could be reduced by 90%, but phytoplankton and bacteria were largely unaffected. This was confirmed by

Mason *et al.* (2003) and Gavand *et al.* (2007) who demonstrated that using ultrasound alone resulted in the mortality of 19 and 49% of bacteria (Mason *et al.*, 2003) and 5 and 33% of the green alga *Dunaliella tertiolecta* (Gavand *et al.*, 2007) after 5 and 20 min respectively. When using ultrasound alone, the decontamination process was shown to be relatively slow using flow-through systems (Mason *et al.*, 2003). Therefore, for effective treatment of ballast water, water would need to be recirculated through the ultrasonic unit or perhaps ultrasound needs to be applied in conjunction with another treatment such as heat, UV or a chemical biocide.

When sonication is combined with chemical treatments the biocidal action can be significantly improved. For example, after 20 min exposure, *Dunaliella tertiolecta* mortality resulting from the individual treatment of sonication, 100 ppm of ozone and 100 ppm of hydrogen peroxide ranged from 33-40%, while 71-81% mortality was achieved when sonication was combined with ozone or hydrogen peroxide (Gavand *et al.*, 2007). Furthermore, a mortality of 100% was achieved following 5 min exposure with the combined treatments of hydrogen peroxide, ozone and sonication. Likewise, the combination of chlorine (1 ppm) and ultrasound reduced bacteria by 86% after 5 min, and 100% after 20 min (Mason *et al.*, 2003), demonstrating that it is possible to combine ultrasound with chemical biocides to achieve an increase in organism removal and/or a reduction in the amount of biocide required for effective treatment. Similarly, Sassi *et al.* (2005) showed that ultrasound treatment alone resulted in the mortality of 80-99% of zooplankton but the combination of ultrasound and UV achieved mortality rates of 97-100%.

To date, the majority of studies investigating the effectiveness of ultrasonic treatment against marine organisms have been conducted using only small volumes of water and low flow rates, typically 5-375 L/min. The up-scaling of ultrasound systems for ballast water treatment may prove problematic as flow rates of a single ballast pump are as high as 5,000 m^3/h. One potential solution would be to recirculate ballast water through the ultrasound device but this may not be possible on short voyages due to time constraints. In addition, recirculating ballast water may also cause ship stability problems, may resuspend ballast tank sediment thus increasing turbidity, and due to the configuration of ballast tanks, some of the ballast water may not be exposed to the ultrasound device (Sassi *et al.*, 2005). Ultrasound decontamination of ballast water

has so far not been tested but is likely to require pre-filtration, as ultrasound is unlikely to penetrate sediments (Rigby and Taylor, 2001; Mason *et al.*, 2003). Pre-filtration may however decrease effectiveness against smaller organisms by removing particulate matter that would increase kills through collision. Overall effectiveness is also influenced by water temperature, water depth and organism concentration. Sassi *et al.* (2005) estimate the cost of an ultrasound ballast water treatment system for a ship with a ballast capacity of around 50,000 tonnes is in the vicinity of US$6 million with an operational cost of approximately $0.56 per tonne of ballast water, but Environmental Technologies Inc. claim to be developing a system costing only US$500,000 with an operational cost of US$0.005 (Lloyd's Register, 2007). Apart from costs, other aspects that require consideration include health and safety issues, which may arise from noise generated by the ultrasound treatment unit, high energy requirements and hull integrity problems due to repeated exposure to ultrasound.

Electrocution

Electrocution has been considered as a potential treatment of ballast water organisms during ballasting and deballasting and was first proposed by Montani *et al.* (1995). In their study, germination of dinoflagellate cysts was inhibited using a dosage of 100 V AC for a period of five seconds. Hallegraeff *et al.* (1997) demonstrated that *A. catenella* cysts were readily killed using a 5 second shock applied to a 4 cm^2 area (>5 V/cm^2); however cysts of *G. catenatum* exhibited a 7% germination success when dosed with a shock of 7.5 V/cm^2 for 5 seconds. No direct comparison can be made between results obtained by these two studies as Montani *et al.* (1995) provide no detailed description of the apparatus utilised or the precise area that the electric shock was applied. The biocidal action of this electrocution technique was claimed to be the generation of chlorine and heat rather than the electric shock itself (Hallegraeff *et al.*, 1997).

The inactivation of bacteria by generating pulsed electric fields has been demonstrated by Blatchley and Isaac (1992) and Aronsson *et al.* (2001). The latter authors examined the killing effect of pulsed electric fields on four microorganisms, including *E. coli*, and found significant reductions do occur (4-to 8-log). Blatchley and Isaac (1992) and Aronsson *et al.* (2001) suggest the reduction of bacteria can be

directly ascribed to the electric treatment, rather to the generation of other chemicals or heat during the process. Treated cells were found to have suffered no membrane damage suggesting that pulsed electric field treatment has profound effects on the intracellular organisation of microorganisms (Aronsson *et al.*, 2001). However, it is possible that the killing action demonstrated in these experiments is due to other chemical reactions occurring as electrical currents are passed through the liquid medium.

Dosing organisms with an electric shock during ballasting would require the electric shock itself to be biocidal. As the production of chlorine has been demonstrated as the biocidal component of electric shock treatment, this would negate electrocution as a ballast water biocide, however electrolysing seawater has been shown to be an economical way of producing free chlorine (e.g. Dang *et al.*, 2004, Matousek *et al.*, 2006).

Mechanical damage

The use of high velocity pumps during ballast water intake and discharge may cause lethal damage to some organisms by mechanical abrasion. Taylor *et al.* (2002) suggest that these systems are hard to install and the cost for installing additional infrastructure to create high velocity jets of water in ballast tanks or pipelines would be prohibitively expensive. Additionally, once water velocity exceeds a certain point (~3 m/s), corrosion problems may become a concern (Taylor *et al.*, 2002). Veldhuis *et al.* (2006) found that the use of a centrifugal water pump with a capacity of 530 m^3/h effectively reduced the total number of living zooplankton, indicating that current ballast water pumps may reduce the survival of larger organisms, but microorganisms, especially bacteria, would be expected to pass through the pump systems with minimal mortality. Hillman *et al.* (2004) tested the effectiveness of a prototype high-velocity sonic/shear disintegrator which generates shear and sonic stresses to destroy organisms. Initial results showed that the device was capable of destroying between 97.7 and 100% of *Artemia* nauplii and reduced the hatching rate of *Artemia* cysts by 47%. Promising research has also been conducted on a mechanical treatment device called the 'Special Pipe System' which terminates

organisms using sheer stress and cavitation produced by the special plate structures of the system. This system is used in conjunction with ozone and is discussed later.

Magnetic treatment

Magnetic treatment has been utilised for the elimination of bacterial growth in diesel fuel. In this process, a magnetic field is pulsed along fuel lines generating very low frequency, non-ionising electromagnetic radiation. This radiation has a much lower frequency than microwave radiation, even lower than radio-waves, yet has a controlling effect on microorganisms (Clearwater Systems Corporation, 2004). However, this technique is considered bacteriostatic rather than bactericidal, as the bacteria are controlled rather than killed. Oemcke (1999) tested a magnetic treatment system on spores of *Bacillus subtilis* and found no effect. No efficacy data are available.

Hitachi have developed an environmentally friendly ballast water treatment system called 'ClearBallast', which treats ballast water by removing target organisms and other objects using flocculating agents and magnetic separation. The system operates by the addition of a magnetic powder, a coagulant and a flocculant, which collide with small organisms and aggregate to form magnetic flocs. The flocs are then separated from the ballast water by magnetic separation technology and are removed in a rotating filter drum resulting in the formation of a concentrated sludge that is temporarily stored in part of the treatment system and must be disposed of in landfill (Fig. 1).

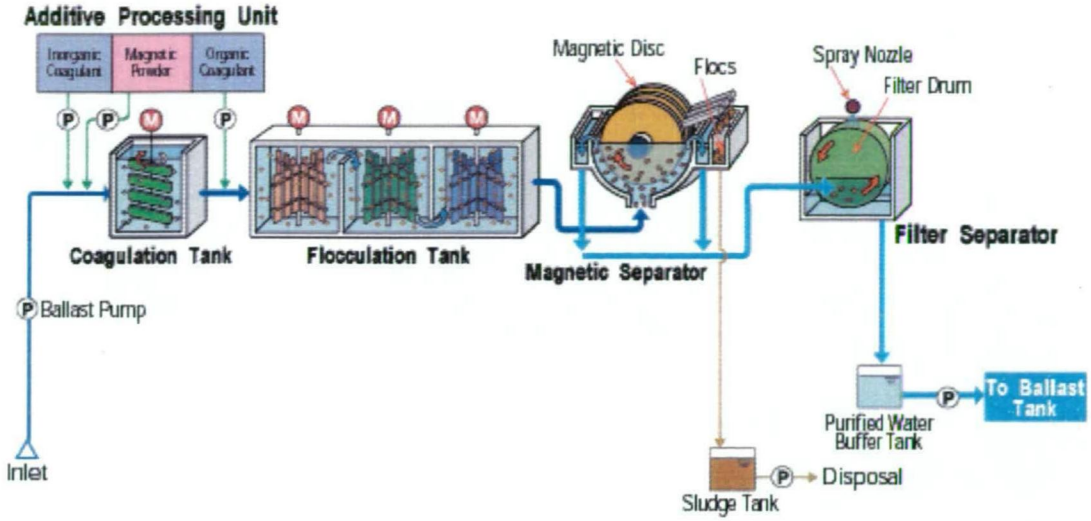

Fig.1. Schematic diagram of the operating processes of the Hitachi 'Clearballast' ballast water treatment system (http://www.mhi.co.jp).

This coagulation magnetic separation method is suggested to remove not only zooplankton and phytoplankton but also bacteria, sediment and suspended solids in the ballast water (Saho *et al.*, 2004). Land-based tests have been conducted on a pilot-scale system at a flow rate of 50 m^3/h using water taken directly from Tokyo Bay. Results of the organism removal efficiency of the system revealed that it was able to meet the IMO's D-2 regulation for the discharge of ballast water. The system removed 100% of organisms greater than 10 μm in dimension, reduced the concentration of *E. coli* from 1,100 to 2 cfu/100 ml and reduced the suspended solid concentration to non-detectable levels (MEPC, 2007b)

The system is considered to be environmentally friendly since most of the three active substances (Triiron tetraoxide, Basic aluminium chloride, Acrylamide sodium acrylate copolymer) that are added to the ballast water are collected in the flocs and removed (MEPC, 2007b). No volatile organic compounds have been identified in treated ballast water but trace amounts of aluminium (0.08 mg/L) were detected (MEPC, 2007b). The use of the 'Clearballast' system on large ships may be limited by space restrictions. The manufacturer suggests that the system can be scaled up or multiple sets of equipment can be installed allowing a commercial system to treat ballast water at flow rates from 50 to 10,000 m^3/h, however the volume of the major components of the system increases in proportion to the flow rate. A treatment system capable of handling 200 m^3/h has a footprint of 20 m^2, while 100 m^2 is required for a system with a capacity of 2,000 m^3/h (Lloyd's Register, 2007). No installation or

operational costs are available at the current stage of development but the system has received Basic Approval from the IMO for the use of active substances.

The above results would suggest that marine microorganisms are not likely to be killed directly by magnetic treatment but the use of systems incorporating magnetic separation and filtration may provide an effective ballast water treatment.

6.4 Chemical treatment options

Many chemical treatment options have been proposed as potential solutions to the problem of ballast-mediated aquatic organism introductions. For a potential chemical treatment option to be effective it must: 1) inactivate all ballast water organisms including resistant resting life stages; 2) not produce toxic by-products; 3) not be hazardous to ships' crew nor corrosive to ships' structures; 4) be cost effective; and 5) degrade at a rate that allows its safe discharge into the aquatic environment. The following section discusses the advantages and limitations of proposed chemical options for the control of ballast water organisms. Table 8 provides a summary of the biological efficacy of potential chemical treatment options.

Table 8. Summary of biological efficacy of proposed chemical ballast water treatment options against aquatic microorganisms.

Treatment Option	Organism	Treatment	Efficacy	Reference
Chlorine	Marine bacteria (free-living)	1-100 ppm, 5 min-24 h	100% mortality	McCarthy and Miller (1994); Sousa *et al.* (2001); Stocks *et al.* (2004b); Zhang *et al.* (2004)
	Marine bacteria (attached to crustaceans)	800 ppm, 5 min	below detection levels	McCarthy and Miller (1994); Sousa et al. (2001); Zhang et al. (2004)
	Bacterial spores	500 ppm	99.9% inactivation	Sagripanti and Bonifacino (1996)
	Vegetative microalgae	1-100 ppm, 24-72 h	100% mortality	Piyatiratitivorakul *et al.* (2002); Stocks *et al.* (2004b); Zhang *et al.* (2004)
	Dinoflagellate cysts (*Gymnodinium catenatum*)	>500 ppm, 24 h	100% inactivation	Bolch and Hallegraeff (1993)
	Zooplankton	2-40 ppm, 24 h	100% mortality	Stocks *et al.* (2004b); Zhang *et al.* (2004)
	Zooplankton resting stages	100-2,500 ppm, 24 h	100% mortality	Stocks et al. (2004b); Gray *et al.* (2006)
- electrolytic chlorine generators	Marine bacteria	3-4 ppm	99.99-99.999% mortality	Dang *et al.* (2004); Matousek *et al.* (2006)
	Phytoplankton	3-4 ppm	72%->99% mortality	Dang *et al.* (2004); Matousek *et al.* (2006)
	Zooplankton	4-15 ppm, 12-24 h	95->99% mortality	Dang *et al.* (2004); Matousek *et al.* (2006)
Chlorine dioxide	Marine bacteria	5-20 ppm, 24 h	100% mortality	Ecochlor (2003); Gregg and Hallegraeff (2007)
	Vegetative microalgae	3-25 ppm, 30-120 min	100% mortality	Junli *et al.* (1997); Gregg and Hallegraeff (2007)
	Dinoflagellate cysts	25 ppm, 2 weeks	100% inactivation	Gregg and Hallegraeff (2007)
	Zooplankton	5 ppm	70-100% mortlity	Junli *et al.* (1997); Oemcke (1999)
	Zooplankton resting stages (*Artemia salina* cysts)	3 ppm, 40 h	97% inactivation	Oemcke (1999)
- chlorine dioxide generators	Marine bacteria (including *V. cholera* and *E. coli*)	5 ppm, 24 h	below detection levels	Ecochlor (2003); Swanson and Perlich (2006)
	Vegetative microalgae	5 ppm 24 h	99.6% mortality	Ecochlor (2003)
	Zooplankton	5 ppm, ≤24 h	98.4-100 % mortality	Ecochlor (2003); Swanson and Perlich (2006)

Table 8. *cont.* Summary of biological efficacy of proposed chemical ballast water treatment options against aquatic microorganisms.

Treatment Option	Organism	Treatment	Efficacy	Reference
Ozone	Marine bacteria (free-living)	>5 ppm Total Residual Oxidants (TRO), 5-10 h	up to 99.99% reduction	Herwig *et al.* (2006); Perrins *et al.* (2006)
		0.15-0.20 ppm (residual ozone), 180 sec	99.99% inactivation	Liltved *et al.* (1995)
	Bacteria (*Bacillus subtilis*)	8-14 ppm TRO, 24 h	complete inactivation	Oemcke (1999)
	Dinoflagellates	>5 ppm TRO, 10 h	>99% reduction	Herwig et al. (2006)
	Microflagellates	>5 ppm TRO, 10 h	96-99% reduction	Herwig et al. (2006)
	Diatoms	>5 ppm TRO, 10 h	17-135% of initial concentrations	Herwig et al. (2006)
	Zooplankton	0.75-7 ppm, 5-48 h	90-100% reduction	Sassi *et al.* (2005); Jones *et al.* (2006); Herwig *et al.* (2006); Perrins *et al.* (2006)
Hydrogen peroxide	Bacterial spores	100,000-140,000 ppm	90-99.99% mortality	Sagripanti and Bonifacino (1996)
	Vegetative microalgae	3-100 ppm, 15 min-48 h	100% mortality	Ichikawa et al. (1993); Piyatiratitivorakul et al. (2002);
	Dinoflagellate cysts	100-10,000 ppm, 24-96 h	100% inactivation	Bolch and Hallegraeff (1993); Ichikawa *et al.* (1993); Montani *et al.* (1995)
	Zooplankton	10 ppm (with increased pH), < 5min	100% mortality	Kurizian *et al.* (2001)
Peracetic acid	Coliform bacteria	6-8 ppm	>97% reduction	Baldry and French (1989)
	Bacterial spores	300 ppm	99.99% mortality	Sagripanti and Bonifacino (1996)
Glutaraldehyde	Bacteria (free-living)	8-14 ppm	83% inhibition	Sano *et al.* (2003)
	Bacterial spores	20,000 ppm	99.99% inactivation	Sagripanti and Bonifacino (1996)
	Zooplankton	11-550 ppm, 24-48 h	90% mortality	Sano *et al.* (2003, 2004)
	Zooplankton resting stages (*Artemia salina* cysts)	353 ppm, 72 h	90% mortality	Sano *et al.* (2004)

Table 8. *cont.* Summary of biological efficacy of proposed chemical ballast water treatment options against aquatic microorganisms.

Treatment Option	Organism	Treatment	Efficacy	Reference
Peraclean® Ocean	Marine bacteria	125-250 ppm	100% mortality	Gregg and Hallegraeff (2007)
	Vegetative microalgae	50-200 ppm, 48 h	100% mortality	Wright *et al.* (2004); Fuchs *et al.* (2001); Gregg and Hallegraeff (2007)
	Dinoflagellate cysts	150-400 ppm, 2 weeks	100% inactivation	Gregg and Hallegraeff (2007)
	Zooplankton	100-400 ppm, <1-72 h	>90-100% mortality	Fuchs and de Wilde (2004); Wright *et al.* (2004); Veldhuis *et al.* (2006); de Lafontaine *et al.* (2008a,b)
	Zooplankton resting stages (*Artemia salina* cysts)	>350 ppm-700 ppm, 72 h	100% inactivation	Fuchs *et al.* (2001); Fuchs and de Wilde (2004)
	Freshwater fish species	100-150 ppm, <19 h	100% mortality	de Lafontaine *et al.* (2008a)
	Fish eggs (*Clupea harengus*)	200-400 ppm, 1-16 h	100% mortality	Fuchs and de Wilde (2004)
SeaKleen®	Marine bacteria	1-200 ppm,	100% mortality	Wright and Dawson (2001); Cutler *et al.* (2004); Gregg and Hallegraeff (2007)
	Vegetative microalgae	0.5-2 ppm, 24-48 h	100% mortality	Wright and Dawson (2001); Cutler *et al.* (2004); Gregg and Hallegraeff (2007)
	Dinoflagellate temporary cysts	2 ppm, 2 h	100% mortality	Cutler *et al.* (2004)
	Dinoflagellate resting cysts	6-10 ppm, 2 weeks (*Alexandrium catenella* not controlled at 10 ppm)	100% inactivation	Gregg and Hallegraeff (2007)
	Zooplankton	0.5-2 ppm, 24-48 h	100% mortality	Cultler *et al.* (2004); Wright *et al.* (2004)
	Zooplankton resting eggs	2.6-12.7 ppm, 24 h	90% mortality	Raikow *et al.* (2006)
Acrolein®	Bacteria	3-10 ppm	>99.99->99.999% reduction	Penkala *et al.* (2004)
	Marine bacteria (onboard trial)	9-15 ppm, 24-72 h	99.99-99.9999% reduction	Penkala *et al.* (2004)
	Phytoplankton	1 ppm	>99.999 reduction	Penkala *et al.* (2004)
	Zooplankton	0.18-0.5 ppm, 96 h	50 % mortality	Penkala *et al.* (2004)
Hydroxyl radical generation	Microorganisms (unicellular algae, protozoans, bacteria)	0.63 mg/L, 2.67-8 sec	100% mortality	Bai *et al.* (2005); Zhang *et al.* (2005)

Table 8. *cont.* Summary of biological efficacy of proposed chemical ballast water treatment options against aquatic microorganisms.

Treatment Option	Organism	Treatment	Efficacy	Reference
De-oxygenation				
- addition of glucose	Zooplankton (*Coscinasterias calamaria)*	>10 mM glucose concentration, 19 d (always >5 ppm O_2)	40% mortality	Mountfort *et al.* (1999)
	Macroalgal zoospores (*Undaria pinnatifida*)	20 mM glucose concentration, 28 d (always >5 ppm O_2)	ineffective	Mountfort *et al.* (1999)
- addition of sulphide	Zooplankton (*Coscinasterias calamaria)*	60 µM sulphide, 68h (always >5 ppm O_2)	100% mortality	Mountfort *et al.* (1999)
	Macroalgal zoospores (*Undaria pinnatifida*)	200 µM sulphide, 36 d (always >5 ppm O_2)	40% mortality	Mountfort *et al.* (1999)
- vacuum chambers	Zooplankton ≥75 µm	< 1 ppm O_2, <3 d	100% mortality	Browning Jr. *et al.* (2004)
- nitrogen sparging	Zooplankton (*Coscinasterias calamaria)*	2.3 ppm , 30 min	99.9% mortality	Mountfort *et al.* (1999)
	Macroalgal zoospores (*Undaria pinnatifida*)	2.8 ppm O_2, 10 min	99.9% mortality	Mountfort *et al.* (1999)
- inert gas sparging (86% N_2, 12% CO_2, 2% 0_2)	Zooplankon	non-detectable O_2, 15 min-48 h	>95% mortality	Husain *et al.* (2004)
	Marine Bacteria (*Vibrio cholerae*)	non-detectable O_2, 24 h	>99% mortality	Husain *et al.* (2004)
- Venturi Oxygen Stripping™	Zooplankton	0.27-<1 ppm O_2, <48-120 h	99-100% mortality	Tamburri *et al.* (2004); NEI Marine (2007)
	Phytoplankton	<1 ppm O_2, 120 h	100% mortality	NEI Marine (2007)
	Bacteria (Enterococci, *E. coli*)	<1 ppm O_2, 120 h	99.9% reduction	NEI Marine (2007)
pH adjustment	Dinoflagellate cysts	pH 2-10	no effect	Bolch and Hallegraeff (1993)
	Mixed zooplankton	pH 8.5-10	no effect	Kurizian *et al.* (2001)
	Ctenophore *Mnemiopsis leidyi*	pH 8.5-10	no effect	Kurizian *et al.* (2001)
Salinity Adjustment	Dinoflagellate cysts	15-50 ‰ salinity	no effect	Bolch and Hallegraeff (1993)
		100 ‰ salinity	prevented germination	

Chlorine

For the past few decades, chlorine has been used as the disinfectant of choice in water treatment technologies due to its cost-effectiveness. Chlorine can be dosed to water in a variety of forms including liquefied chlorine gas, sodium hypochlorite, calcium hypochlorite, or can be generated electrolytically from seawater. The broad-spectrum biocidal activity of chlorine is mediated by hypochlorous acid, which is formed in aqueous solutions at pH 5-8 (Gardner and Peel, 1991). The toxicity of chlorine is a function of several factors including chlorine concentration, pH, exposure time, and type and quantity of chlorine compounds formed.

Chlorination has been shown to eliminate aquatic organisms but the concentration required varies considerably with different organisms. Vegetative algal cells and free-living zooplankton can be killed at concentrations of 1-100 ppm (Laughton *et al.,* 1992; Piyatiratitivorakul *et al.,* 2002; Stocks *et al.,* 2004b; Zhang *et al.,* 2004); however resistant organisms, such as dinoflagellate cysts, zooplankton resting stages and *Bacillus subtilis* spores require considerably higher concentrations (486-2,500 ppm) (Bolch and Hallegraeff, 1993; Stocks *et al.,* 2004b; Gray *et al.* 2006).

Bacterial susceptibility to chlorine varies greatly, with free-living gram-positive and gram-negative bacteria highly susceptible, whereas acid-fast bacteria, bacteria associated with crustaceans and bacterial spores require higher doses. For example, *Bacillus subtilis* spores require a chlorine concentration of 500 ppm for inactivation to occur (Sagripanti and Bonifacino, 1996), whereas Zhang *et al.* (2004) completely eliminated *Vibrio* sp. at 5 ppm. Similarly, Sousa *et al.* (2001) found doses of 10 ppm were effective in killing free-living *Vibrio cholerae*; however this concentration was insufficient to destroy *Vibrio's* adhering to crustaceans. It has been suggested that the presence of crustaceans causes an increase in organic matter, reducing the bactericidal properties of chlorine (Sousa *et al.,* 2001). Another study found toxigenic strains of *V. cholerae* required a much higher dose of 100 ppm for the control of free-living cells, with even higher doses (800 ppm) required to achieve satisfactory control of attached *V. cholerae*, yet even at this concentration, regrowth was apparent within 20 min (McCarthy and Miller, 1994). Chlorination at 800 ppm was not effective against *V. cholerae* cells in samples of undiluted or 10% ballast water suggesting that

attachment to organisms and particulate matter in the ballast water enhanced their survival (McCarthy and Miller, 1994).

The biocidal activity of chlorine may be increased if combined with other treatment technologies such as heat or ultrasonic treatment. Mason *et al.* (2003) found the lethality of ultrasound on bacterial growth doubled after 20 mins when combined with chlorine at a concentration of 1 ppm. This would also act to drastically reduce the treatment cost.

Chlorine concentrations required for the effective treatment of ballast water are likely to be prohibitively expensive. Bolch and Hallegraeff (1993) indicate that the costs required for adequate chlorination of 50,000 tonnes of ballast water would be in the vicinity of A$200,000. Electrolysed seawater may be used to generate chlorine for shipboard ballast water treatment (Dang *et al.*, 2004; Matousek *et al.*, 2006). Matousek *et al.* (2006) found that a 3 ppm chlorine concentration generated electrolytically from seawater reduced bacteria by more than 99.999% and reduced phytoplankton and mesozooplankon by 99%. Conversely, Dang *et al.* (2004) found that only 72% of total phytoplankton can be killed if raw seawater is treated by electrolysis with an initial chlorine concentration of 4 ppm. Electrolysing seawater may act to reduce the cost of chlorine treatment; however, several other factors negate the use of chlorination for the control of organisms in ships' ballast water. Foremost, there is significant concern over the creation of toxic organo-chlorides and subsequent environmental impacts at the port of ballast discharge. A shipboard assessment of the use of chlorine for ballast water treatment conducted by Vianna da Silva and da Costa Fernandes (2004) found that chlorine concentrations above 3 ppm should not be used due to the formation of high concentrations of toxic by-products such as carcinogenic trihalomethanes (THM) and haloacetic acids (HAA). These products are formed when chlorine reacts with naturally occurring organic and inorganic matter and levels tend to increase with pH, temperature, time and quantity of organic matter. Once discharged in ballast water, these products can persist in the marine environment, bioaccumulate in the food chain and can interfere with or destroy the hormonal systems of marine organisms (Jenner *et al.*, 1997). Additionally, ballast tank sediment is likely to reduce the available free chlorine levels (Bolch and

Hallegraeff, 1993; Gray *et al.*, 2006); and chlorine treatment has been claimed to increase corrosion of the ship (Stocks *et al.*, 2004b; Zhang *et al.*, 2004).

Chlorine dioxide

Chlorine dioxide (ClO_2) is not used widely in wastewater disinfection due to the high cost involved compared with chlorination. However, the use of ClO_2 in ballast water disinfection is considered advantageous over chlorine for several reasons. Chlorine dioxide treatment is not particularly reactive with organic material, can maintain the bactericidal and inactivation effects within a wider pH range than chlorine and is considered to be more environmentally friendly than chlorine as it does not involve or create free available chlorine or chlorinated by-products. (Muntisov *et al.*, 1993; Junli *et al.*. 1997; Vianna da Silva and da Costa Fernandes, 2004).

The majority of ClO_2 work has been conducted on freshwater organisms (e.g. Korich *et al.*, 1990; Lykins Jr. *et al.*, 1994; Junli *et al.*, 1997), with only few studies available for marine systems (Hillman, *et al.*, 2004; Gregg and Hallegraeff, 2007). Nonetheless, results indicate that it is an excellent bactericide and sporicide, and is more effective in controlling vegetative bacteria and viruses compared with chlorine. For example, a range of viruses could be controlled at a concentration of 1-7 ppm ClO_2, whereas a 7 ppm dose of chlorine had no observable effects (Junli *et al.*, 1997). Yet, Junli *et al.* (1997) also found that there was no significant difference in the inactivation effect on algae between ClO_2 and chlorine, but concludes that ClO_2 is better than or matchable to liquid chlorine in the killing effect on algae.

Efficacy studies conducted in seawater have found that vegetative cells of the dinoflagellates *Alexandrium catenella, Gymnodinium catenatum, Protoceratium reticulatum* and *Scrippsiella trochoidea* are eliminated at 25 ppm after 2 h; and sexual resting cysts of *G. catenatum* and *P. reticulatum* are inactivated following 2 weeks exposure to 50 ppm (Gregg and Hallegraeff, 2007) Hillman *et al.* (2004) tested the effectiveness of a chlorine dioxide generator against *Artemia* cysts and concluded that a 3 ppm concentration can reduce the hatching rate by 97%.

Apart from high costs, the production of harmful by-products from ClO_2 is considered the greatest concern. The major by-products resulting from chlorine dioxide disinfection are chlorite, chlorate, and organic, biodegradable by-products such as carbonyl compounds and short chain carboxylic acids (Raczyk-Stanislawiak *et al.,* 2004). Chlorate has been documented to be toxic to marine microalgae, particularly in nitrate-limited waters (Stauber, 1998), however these toxic chlorate concentrations are unlikely to result from the discharge of ballast water.

Newer ClO_2 generators are suggested to produce no chlorinated by-products or toxic residual material at discharge. The Ecochlor® Ballast Water Treatment System generates chlorine dioxide using the Eka Chemical Purate® technology, a method that differs from conventional chlorine dioxide generation methods in that it does not involve or create free available chlorine or chlorinated by-products. The treatment system uses Purate® solution (containing 40% sodium chlorate and 8% hydrogen peroxide) and sulfuric acid to produce ClO_2, which is injected into ballast water during intake.

Laboratory experiments demonstrated that an initial ClO_2 concentration of 5 ppm was effective at eliminating bacterial and planktonic populations to the extent that no regrowth was observed (Swanson and Perlich, 2006). Shipboard studies on the biological efficacy of the treatment system as well as degradability and corrosion tests were carried out onboard the MV *Atlantic Compass* in 2005. Results from these experiments showed that the application of 5 ppm of ClO_2 immediately eradicated 100% of zooplankton, reduced the abundance of total coliform bacteria, *Vibrio* colonies and *E. coli* to non-detectable levels within the first 24 h, and virtually eliminated all phytoplankton biomass (Swanson and Perlich, 2006). Some recovery of bacteria and phytoplankton was observed after 5 days indicating that the treatment did not eliminate 100% of organisms. This regrowth was attributed to the presence of a biofilm in the ballast tanks which provided refuge for the organism that survived (Swanson and Perlich, 2006).

Degradability studies conducted during the shipboard trials determined that ClO_2 remained active in the ballast tanks for several hours but degraded at a rate that resulted in no residual ClO_2 in the ballast water at the time of discharge. For example,

an initial dosage of 5 ppm left no residual ClO_2 after 20-24 h when the temperature of ballast water was between 10 and 12°C, whilst an initial dosage of 8 ppm in 25-26°C ballast water left no trace after 24-25 h (Swanson and Perlich, 2006). Furthermore, corrosion tests conducted in ballast tanks indicated that ClO_2 had no adverse effect on corrosion, in fact a slight decrease in corrosion was observed in the treated tanks during one experiment (MEPC, 2008a). It should be noted that the corrosion tests were only carried out for a period of 28-32 days. Much longer investigations are required to fully understand the effect of the treatment system on corrosion rates.

Advantages of the Ecochlor® Ballast Water Treatment System are that it is able to operate effectively under extremely varied water quality conditions at any temperature or salinity without the need for filtration or pre-treatment of the ballast water. It also has the capacity to treat ballast water at high flow rates. The system does not require the storage of large amounts of active substances onboard but does require the storage of the pre-cursor chemicals. According to Carney *et al.* (2008), the capital cost of the system is between US$260,000 and US$400,000 for flow capacities of 200 m^3/h and 2000 m^3/h, respectively, with an operating cost of US$0.06 per tonne of ballast water.

In summary, it appears that ClO_2 is toxic to a wide range of aquatic organisms and should be considered as a ballast water treatment. Indications are that it is more environmentally friendly than chlorine as degradation products are not expected to persist or bioaccumulate in the marine environment. Further work is required to assess the ability of the biocide to inactivate organisms in ballast tank sediment and possible corrosion impacts.

Ozone

Ozone is a powerful oxidant used to control microorganisms in a variety of applications. It has been demonstrated to be an excellent sterilisation agent in freshwater systems (Korich *et al.,* 1990). However, the biocidal activity of ozonated seawater may differ considerably from that of ozonated freshwater as ozone reacts with bromide and chloride ions to form oxidants in seawater (Sugita *et al.,* 1992b).

Ozone is an unstable gas that decomposes readily to oxygen, therefore must be generated on-site. Ozone can be safely generated from air and could prove beneficial over many other chemical treatment options for several reasons. Firstly, there is no need to store large amounts of chemicals onboard ships and the chemical does not need to be physically dosed into ballast tanks by crew. Secondly, ozone is considered advantageous over other chemical oxidants, as it poses fewer environmental problems. Toxic by-products created during the ozonation of seawater (e.g. brominated organics, bromate, bromamine) can cause environmental impacts at low concentrations, however, concentrations required to impact on most organisms are considerably higher than those generated from ozonation (Stewart *et al.*, 1979).

Ozone reacts with bromide in seawater resulting in a half-life of 5.3 seconds (Andersen *et al.*, 1995), nonetheless it is possible that molecular ozone will make some contribution to disinfection in seawater regardless of its reactions with bromide and chloride, however, the majority of disinfection is believed to be due to the production of hypobromous acid/hypobromite (Perrins *et al.*, 2006b), which are generally measured as total residual oxidants (TRO's).

The ozonation of seawater has been shown to successfully remove bacterial fish pathogens and viruses at low concentrations with low exposure times (Sugita *et al.*, 1992b; Liltved *et al.* 1995). Sugita *et al.* (1992b) propose that ozone treatment at a total residual oxidant (TRO) concentration of more than 1 ppm for several minutes is able to disinfect seawater, while Liltved *et al.* (1995) demonstrated that a concentration of 0.10-0.20 ppm of residual ozone inactivated all tested viral and fish pathogens within 3 min. Perrins *et al.* (2006a) found that although culturable bacteria were initially controlled at TRO concentrations of >1 ppm, once TRO fall below the bacterial inhibition threshold of ~0.5 ppm, heterotrophic bacteria rapidly grow.

Results from several laboratory studies suggest that some organisms are resistant to ozone. For example, Andersen *et al.* (1995) assessed ozone treatment using several planktonic algae and crustaceans as model organisms. All test organisms were effectively controlled by ozonation but dosage required varied between organisms. Dosages required to inactivate crustaceans were found to be 2-5 times greater than those needed to inactivate algal species such as *Amphidinium carterae* and

Karlodinium veneficum (=*Gymnodinium galatheanum*) (Andersen *et al.,* 1995). Experiments conducted on *Bacillus subtilis* found that much higher TRO dosages of up to 14 ppm are required for adequate inactivation at a pH of 8 with a 24 hr contact time, however reducing the pH to 7 resulted in a reduced dose of 9 ppm required for disinfection (Oemcke, 1999). Other organisms that have been demonstrated to be resistant to ozone treatment include dinoflagellate cysts, Cirripeda nauplii, Polychaeta larvae, copepod nauplii, cyclopoid and harpacticoid copepods, mysid shrimp and shore crabs (Deacutis and Ribb, 2002; Herwig *et al.,* 2006; Perrins *et al.,* 2006a).

Several companies have produced onboard treatment systems that inject ozone into ships' ballast water during ballast intake; however doubts exist as to whether these systems cost-effectively control all organisms. For example, mesocosm and shipboard testing of the biological efficacy of the Nutech O3 ozone generator found that the system was capable of significant reductions of up to 99.99% of bacteria, >99% of dinoflagellates, 99% of microflagellates and zooplankton after 5-10 h treatment when the TRO levels exceeded 5 ppm (Herwig *et al.,* 2006; Perrins *et al.,* 2006a). However these reductions varied considerably between experiments and between sampling locations within the experiments as the distribution of ozone was not homogenous (Herwig *et al.,* 2006). The treatment system was much less effective against diatoms and a number of crustacean species (Herwig *et al.,* 2006; Perrins *et al.,* 2006a). The experiments did not assess treatment effects on resting stages of zooplankton or phytoplankton.

The above results raise doubts to whether ozone treatment can cost-effectively control all ballast water organisms as many organisms, especially bacterial spores, dinoflagellate cysts and many zooplankton species, are likely to survive ozone treatment at the concentrations that are considered environmentally safe and achievable in onboard situations. Ozone may provide an effective control option for vegetative microalgae, pathogenic bacteria and viruses present in ballast water, though a fine filtration pre-treatment would be necessary to remove resistant organisms. Contact times necessary for bacterial control indicate that ballast tanks would need to be continuously dosed with ozone creating occupational health and safety problems for the crew. Also, iron in ships' structures is likely to be oxidised by ozone and hypobromous acid increasing corrosion (Oemcke and van Leeuwen,

1998). Projected costs of an ozone treatment system are in the vicinity of US$800,000 to $1.6 million with an operational cost of US$0.28-0.32 per tonne of ballast water (Sassi *et al.*, 2005; Carney *et al.*, 2008).

Hydrogen peroxide

Hydrogen peroxide is an oxidative biocide considered attractive for the treatment of ballast water as it is known to be of limited risk to humans and decomposes rapidly resulting in harmless by-products of oxygen and water. It is currently used as a disinfectant in a variety of applications including laboratory and medical applications, and even as a replacement to chlorine for the treatment of swimming pools.

Reported hydrogen peroxide concentrations required for the elimination of marine organisms range from 3 ppm for vegetative dinoflagellate cells of *Karenia mikimotoi* to over 140,000 ppm for *B. subtilis* spores (Ichikawa *et al.*, 1993; Sagripanti and Bonifacino, 1996). A large discrepancy exists in the literature in relation to the concentration required to eliminate vegetative algal cells and dinoflagellate cysts. Dinoflagellate cyst inactivation has been achieved with doses between 100 and 10,000 ppm (Bolch and Hallegraeff, 1993; Ichikawa *et al.*, 1993; Montani *et al.*, 1995; Hallegraeff *et al.*, 1997). This discrepancy may be due to different resistances of isolates utilised in these experiments, different organic loadings of seawater or different brands of hydrogen peroxide may vary in potency. Similarly, different exposure times used in the experiments may explain this inconsistency. For example, Piyatiratitivorakul *et al.* (2002) reported that a concentration of 100 ppm for 24 h resulted in 40 and 66.6% mortality in the raphidophytes *Heterosigma akashiwo* and *Chattonella marina* respectively, whereas a 30 ppm (*H. akashiwo*) and 10 ppm (*C. marina*) for 48 h resulted in the complete mortality. This would suggest that the exposure time is very important aspect of the biocidal action of hydrogen peroxide. Kuzirian *et al.* (2001) found that elevating the pH of ballast water reduced the hydrogen peroxide concentrations required (1 ppm) to eliminate a range of invertebrate taxa including the ctenophore *Mnemiopsis leidyi,* the hydrozoan *Pennaria* sp., and a range of polychaetes, crustaceans, chordates and larval bivalves. Similarly, Mesbahi (2004) suggest that the efficiency of hydrogen peroxide can be enhanced by elevated temperature (up to 35°C).

Bacterial spores are the most resistant marine organisms to hydrogen peroxide treatment requiring doses upward of 100,000 ppm for mortality to occur (Sagripanti and Bonifacino, 1996). The high concentrations needed for spore and dinoflagellate cyst inactivation would exclude hydrogen peroxide as a ballast water treatment as the cost involved are likely to be substantial. Additional concerns include hazards associated with the distribution, handling and onboard storage of large volumes of hydrogen peroxide. Hydrogen peroxide can however be produced *in-situ* by means of an electrochemical conversion of dissolved oxygen, which is carried out in a specially designed and patented electrochemical reactor. The PeroxEgen™ onboard hydrogen peroxide water treatment system is claimed to be able to control ballast water organisms (including bacteria) by injecting low hydrogen peroxide concentrations (<100 ppm) into ballast water during intake (Eltron Water Systems, 2007), however efficacy data are not available. If this method can safely and effectively produce hydrogen peroxide concentrations able to inactivate resistant organisms, it may prove a feasible ballast water treatment.

Glutaraldehyde

Glutaraldehyde is an organic biocide proposed for the treatment of vessels carrying little or no ballast to control organisms present in ballast tank residues and sediment (Sano *et al.,* 2003; 2004). It has been demonstrated to successfully control the marine bacterium *Vibrio fischeri* at a concentration of 8-14 ppm; however doses of 20,000 ppm are required to inactivate *B. subtilis* spores (Sano *et al.,* 2003; Sagripanti and Bonifacino, 1996).

Glutaraldehyde is most active at higher temperatures and above a pH of 7.5 (Sagripanti and Bonifacino, 1996). As ballast tank pH generally varies from 4.2 to 8.6, this may limit the application of glutaraldehyde, unless the treatment system can account for this strong pH response. Glutaraldehyde is corrosive in its concentrated form, but is not considered to pose any corrosion problems in the diluted form proposed for ballast water treatment.

Observations by Sano *et al.* (2003) indicate that some ballast water organisms may be resistant to glutaraldehyde treatment; consequently eliminating most organisms may require a concentration of 500 ppm. Pre-treatment of fine filtration may act to reduce this concentration as the amount of sediment and organic carbon present in ballast tanks is likely to impact on the efficacy of glutaraldehyde. In situations where higher amounts of sediment exist in relation to water, the ability of glutaraldehyde to penetrate these sediments and kill any viable organisms is likely to be limited (Sano *et al.,* 2003). In this situation, even higher doses may be required for adequate organism removal.

The high glutaraldehyde concentration required to control ballast water organisms induces three important limitations with respect to ballast water treatment. Firstly, the cost of treatment will be prohibitively expensive; secondly, the potential for detrimental environmental impacts is increased; and finally, occupational health and safety risks increase. Sano *et al.* (2003) suggest the cost of glutaraldehyde treatment would equate to US$25 per tonne of ballast water, thus limiting the treatment to vessels with small quantities of ballast water and sediment. The increased risk of environmental impacts will relate to the time required for glutaraldehyde to degrade and the amount of dilution that takes place prior to release into the receiving port (Leung, 2001; Sano *et al.,* 2003). The biodegradation of glutaraldehyde relies on its digestion by microbes. As glutaraldehyde is a biocide, the concentration required to remove ballast water organisms is also likely to inhibit bacterial growth and metabolism, however once discharged the concentration will decline as the ballast water is dispersed into the marine environment. The biodegradability of glutaraldehyde in seawater has been tested and is a complex issue varying with factors such as initial concentration, nutrient status and microbe concentration. Leung (2001) indicates that glutaraldehyde is considered readily biodegradable in the freshwater environment and has the potential for biodegradation in the marine environment.

Peracetic acid and Peraclean® Ocean

Peracetic acid is another organic biocide suggested as a potential ballast water treatment due to its broad-spectrum activity and lack of undesirable by-products. Very little data exists on the efficacy of peracetic acid in marine systems; however it has been documented to control coliform bacteria in sewage sludge at a concentration of 6-8 ppm, and bacterial spores at 300 ppm (Baldry and French, 1989; Sagripanti and Bonifacino, 1996). Activity is not affected in the presence of suspended solids and organic matter, however is affected strongly by pH. Sagripanti and Bonifacino (1996) found the biocide to be most active at a pH of less than 3, while activity is lost above a pH of 8. This finding may limit the application of peracetic acid as a ballast water biocide, however Degussa AG of Germany has developed a ballast water treatment product composed of peracetic acid and hydrogen peroxide, with the trade name Peraclean® Ocean. It is suggested to be effective against a broad range of microorganisms including bacteria, spores, phytoplankton, aquatic invertebrates and fish eggs at concentrations of 50-400 ppm and exposure times of 2-72 h (Fuchs *et al.*, 2001; Fuchs and de Wilde, 2004). Apart from broad toxicity, Peraclean® Ocean is claimed to be effective over a wide range of conditions, to be relatively unaffected by organic matter and readily biodegradable and decompose into acetic acid, oxygen and water. The manufacturer indicates that Peraclean® Ocean has a half-life of only 4 h in unfiltered seawater and recommends a retention time of only 1-2 days in ballast tanks due to its rapid degradation. One of the added advantages over peracetic acid disinfection is that it is most active at pH values of 5-7, but displays good activity up to a pH of 9 (Fuchs *et al.*, 2001).

Peraclean® Ocean has been developed as either a stand alone treatment or as the final stage of a treatment system that uses a combination of technologies. Lab-scale testing has shown that the product is capable of eliminating bacteria, vegetative marine microalgae, dinoflagellate cysts and several different development stages of the brine shrimp *Artemia salina*. Gregg and Hallegraeff (2007) eliminated vegetative cells of the marine dinoflagellates *Alexandrium catenella, Gymnodinium catenatum, Protoceratium reticulatum* and *Scrippsiella trochoidea* at 50 ppm and killed the green flagellate *Tetraselmis suecica* at 100 ppm after 48 h exposure. Fuchs *et al.* (2001)

killed the green alga *Chlorella* within 48 h at 200 ppm, however higher concentrations (up to 1600 ppm) did not result in more rapidly mortality (Fuchs *et al.*, 2001). This would suggest, as for hydrogen peroxide, that exposure time is a very important aspect of the biocidal action of Peraclean® Ocean for the control of algae. In addition, Peraclean® Ocean inhibited bacterial regrowth of *Escherichia coli, Staphylococcus aureus, Listeria innocua* and *Vibrio alginolyticus* at 125-250 ppm and could completely inactivate *Artemia salina* cysts and resting cysts of the marine dinoflagellates *Gymnodinium catenatum, Alexandrium catenella* and *Protoceratium reticulatum* at 350-700 ppm (Fuchs and de Wilde, 2004; Gregg and Hallegraeff, 2007)

Full-scale shipboard and land-based testing has proved that Peraclean® Ocean is an effective biocide for the control of a wide range of planktonic organisms and marine bacteria in both freshwater and seawater (Wright *et al.*, 2004; Veldhuis *et al.*, 2006; de Lafontaine *et al.*, 2008a). Veldhuis *et al.* (2006) examined the effectiveness of a full-scale land-based ballast water treatment system that used Peraclean® Ocean as a final disinfection step. Peraclean® Ocean was applied to estuarine water at a concentration of 150 ppm and resulted in the elimination of all zooplankton and phytoplankton but bacterial regrowth was observed after 6 to 10 days indicating that the biocide did not result in full sterilisation (Veldhuis *et al.*, 2006). De Lafontaine *et al.* (2008a) treated freshwater ballast onboard the MV *Canadian Prospector* using a Peraclean® Ocean concentration of ~100-150 ppm and observed a >90% reduction in free-floating microorganisms and phytoplankton after 5 days. The treatment also showed a lethal effect on fish with 100% mortality achieved in less than 19 h for a range of cold water fish species, however the treatment was found to be ineffective against adult zebra mussels and some organisms buried in ballast sediments were not affected by a 5 day exposure to ~100-150 ppm of Peraclean® Ocean (de Lafontaine *et al.*, 2008a).

Although the manufacturer indicates that Peraclean® Ocean-treated ballast water may be safely discharged after 1-2 days, degradability studies have shown that this biocide may degrade at a slow rate resulting in the discharge of potentially toxic ballast water into the marine environment. Results from the shipboard trials conducted by de Lafontaine *et al.* (2008a) suggest a retention time of 15-29 days is required prior to

discharge when using Peraclean® Ocean at a concentration of 100-150 ppm for treating freshwater ballast due to the presence of toxic residues, and studies conducted in marine harbour water recommend a retention time of >6 days (Veldhuis *et al,* 2006). Biodegradability studies by Gregg and Hallegraeff (2007) found that 200 ppm Peraclean® Ocean concentrations degraded to a level non-toxic to marine microalgae in 2-6 weeks. Degradation occurred more rapidly when exposed to light and ballast tanks sediments, whereas filtered seawater, humus-rich seawater, relatively clear freshwater and a lack of light appear to be the worst conditions for the degradation of Peraclean® Ocean (Gregg and Hallegraeff, 2007; de Lafontaine *et al.,* 2008b).

The cost for this type of treatment is suggested to be in the vicinity of US$0.20-0.30 per tonne of ballast water. Apart from been exceedingly expensive for use onboard ships with large ballast capacities, additional factors that may limit the use of Peraclean® Ocean as a routine ballast water treatment options include possible residual toxicity of treated ballast water, reduced effectiveness in the presence of sediments, health and safety issues relating to the need to store large amounts of noxious chemicals onboard and possible ship corrosion.

SeaKleen®

SeaKleen® is patented biocide developed by Garnett, Inc. Atlanta and manufactured by Vitamar Inc. Memphis. It consists primarily of menadione (a water-soluble form of Vitamin K3), which belongs to the chemical class of naphthoquinones, and has been shown to be toxic to a wide range of freshwater and marine organisms. Apart from its broad toxicity, the manufacturer suggests that SeaKleen® is an attractive ballast water biocide because it is apparently of low toxicity to mammals, birds and species of higher fish, it has a short half-life causing it to degrade to harmless products within days, it has no known corrosive properties, and it is relatively cost-effective (Wright and Dawson, 2001). Laboratory testing has proved SeaKleen® to be toxic to aquatic algal species (*Chlorella* sp., *Isochrysis galbana*, *Neochloris* sp., *Tetraselmis suecica*), vegetative dinoflagellates (*Alexandrium catenella, A. tamarense, Gymnodinium catenatum, Karenia brevis*, *K. brevisulcata, Karlodinium veneficum, Prorocentrum minimum, Protoceratium reticulatum, Scrippsiella*

trochoidea), dinoflagellate temporary cysts (*Glenodinium foliaceum*), raphidophytes (*Chattonella marina*) and zooplankton (*Crassostrea virginica* larvae, *Cyprinodon variegates, Dreissena polymorpha* larvae, *Leptocheirus plumulous, Mytilus galloprovincialis*) at concentrations ranging from 0.5 to 2 ppm (Wright and Dawson, 2001; Cutler *et al.,* 2004; Gregg and Hallegraeff, 2007).

Full-scale shipboard trials of SeaKleen® were conducted onboard the USS *Cape May* in Baltimore Harbour in 2001. Results from the tests indicate that dosing ballast tanks with a concentration of 2 ppm SeaKleen® resulted in overall zooplankton mortalities of 99 and 100% after 24 and 48 h (Wright *et al.,* 2004). Phytoplankton were controlled with SeaKleen® concentrations as low as 1 ppm after 24 h, however the effectiveness on bacteria was not clear (Wright *et al.,* 2004, 2007a). Some disagreement exists regarding the bactericidal properties of the product. Wright and Dawson (2001) and Cutler *et al.* (2004) suggest that SeaKleen® is extremely effective against bacteria and can eliminate *Escherichia coli* and *Vibrio fisheri* at a concentration of 1 ppm. Conversely, mesocosm experiments conducted at the University of Washington claimed that SeaKleen® at 2 ppm had no observable effect on culturable bacteria (Herwig and Cordell, 2004) and Gregg and Hallegraeff (2007) required concentrations of 50-200 ppm to inhibit regrowth of *E. coli, Listeria innocua, Staphylococcus aureus* and *Vibrio alginolyticus.*

Inconsistencies also exist in the literature concerning the degradability of SeaKleen®. Herwig and Cordell (2004) and Wright *et al.* (2007a) reported a half life of 18-30 h for SeaKleen®, yet Cutler *et al.* (2004) found that SeaKleen® degraded to only 21% of the initial concentration in darkness in seawater without any organisms after 28 days. The latter authors suggest that degradability is faster under light conditions and in the presence of biological material; however, Gregg and Hallegraeff (2007) found that the degradation of 4 ppm SeaKleen® was minimal after 15 weeks and was not influenced by the presence of ballast tank sediment, biological matter or light conditions. Faimali *et al.* (2006) also report that exposure to light failed to accelerate the degradation rate of SeaKleen®. These authors indicate that a 10 ppm concentration of menadione in drinking water has a half life of 1,500 h under both light and dark conditions and takes >5,000 h to totally degrade.

It has been suggested that the use of SeaKleen® may be advantageous in situations where water turbidity is high or to treat residual ballast tank sediments due to a low binding affinity to particulate matter (Wright *et al.,* 2007a). Several studies indicate that SeaKleen® does retain its activity in the presence of sediment but it is less effective against resistant resting stages and sediment dwelling organisms. For example, Gregg and Hallegraeff (2007) found that the biocidal effect of SeaKleen® was not influenced by the presence of sediment but the product failed to kill resistant resting cysts of the toxic dinoflagellate *Alexandrium catenella* at 5 times the recommended dose (10 ppm) in sediment-free trials. Additionally, Sano *et al.* (2004) controlled the amphipod *Hyalella azteca* at comparable SeaKleen® concentrations in both sediment-free samples (2.5 ppm) and samples containing a 1:4 sediment to water ratio (3.5 ppm), but 88 ppm was required to control the burrowing oligochaete *Lumbriculus variegates* in the 1:4 sediment to water ratio compared to 1.8 ppm required in the water-only exposures. These findings indicate that SeaKleen® may provide an effective treatment against organisms in the water column when ballast water contains a high suspended sediment load. While effective control of resistant resting stages and sediment-dwelling organisms might be possible, the required concentrations would be likely to make the treatment prohibitively expensive and may pose environmental problems due to the discharge of toxic ballast water and residual sediment.

The estimated cost of SeaKleen® is approximately US$0.20 per tonne when applied at a concentration of 2 ppm, which may limit the use of this biocide to vessels with small or moderate ballast capacities. Additional concerns that may limit the use SeaKleen® as a routine ballast water treatment option include the possible discharge of toxic ballast water due to low degradability of the biocide and the limited effectiveness against bacteria.

Several other naphthoquinone compounds are currently being investigated as potential ballast water biocides. These include juglone, plumbagin and menadione nicotinamide bisulphite (Faimali *et al.,* 2006; Wright *et al.,* 2007b). Like menadione, juglone and plumbagin are natural plant products, with juglone isolated from the black walnut tree *Juglans nigra,* and plumbagin, a compound found in members of the sea lavender family, Plumbaginaceae (Wright *et al.,* 2007b). Both products have

been shown to exhibit greater bactericidal activity and overall toxicity to aquatic organisms compared to menadione (SeaKleen®), however menadione was considered to be favourable biocide for ballast water treatment as the production cost of menadione is less than 2% the cost of either juglone or plumbagin (Wright *et al.*, 2007a). Nonetheless, further work should assess the degradability of juglone and plumbagin and their ability to inactivate resistant marine organisms, such as dinoflagellate resting cysts.

Menadione nicotinamide bisulphite is a synthetic derivative of menadione (Faimali *et al.*, 2006). Preliminary screening of its efficacy against marine organisms found that it can effectively eliminate a variety of ballast water organisms in the absence of light. Zooplankton larvae, including *Artemia salina* nauplii, *Balanus amphitrite* nauplii, *Mytilus galloprovincialis* larvae and *Tigriopus fulvus* larvae were completely eliminated at concentrations of 0.5 to 5 ppm, growth of the green alga *Chlorella minutissima* was inhibited at 0.5 ppm and germination of dinoflagellate cysts (*Scrippsiella trochoidea*) was reduced to 30% (compared to 90% in controls) after exposure to 5 ppm (Faimali *et al.*, 2006). The product displayed a variable effectiveness against bacteria (1 to >64 ppm required to inhibit regrowth) and was not as effective against *Alexandrium catenella* (EC 50=32 ppm) (Faimali *et al.*, 2006). The major advantage this compound has over its parent molecule (menadione) is that it is highly biodegradable. Faimali *et al.* (2006) imply that menadione nicotinamide bisulphite has a half life of 48 h under dark conditions and <6 h under light conditions compared to 1,500 h for menadione when prepared in drinking water.

Acrolein®

Acrolein® is broad-spectrum biocide produced by the Baker Petrolite Corporation. It is used extensively in the petroleum industry as a biocide to mitigate bacteria in produced fluids and is sold as an aquatic herbicide to control submerged plants and algae in irrigation canals. It is claimed to be toxic to a range of microorganisms including bacteria and algae, as well as macroorganisms such as molluscs, crustaceans, fish, and aquatic plants (Penkala *et al.*, 2004). Lab-scale experiments on marine organisms indicate that vegetative dinoflagellates and both Gram-negative and Gram-positive spore-forming and non-sporulating marine bacteria can be controlled

at concentrations of 1-6 ppm after contact times of 24 and 72 h (Penkala *et al.,* 2004). After 24 h exposure, a concentration of 6 ppm caused a >99.99% reduction in bacterial strains of *Pseudomonas fluorescens, Bacillus cereus, B. subtilis* and *Staphylococcus epidermidis,* while 10 ppm achieved a >99.999% reduction. No viable motile cells of the marine dinoflagellate *Akashiwo sanguinea* (=*Gymnodinium sanguineum*) were observed at 1 ppm (Penkala *et al.,* 2004). Results from a 5-day shipboard trial found that ballast tanks treated with 9 ppm effectively reduced bacteria by 99.99% for a period of 2 days; and 15 ppm inhibited bacterial regrowth for 3 days, whereas 1 and 3 ppm of Acrolein® was ineffective (Penkala *et al.,* 2004). This would indicate that the demand for Acrolein® in ballast tanks is much higher than what was predicted from laboratory experiments, nonetheless it is estimated that microorganism regrowth can be inhibited by maintaining ≥2 ppm residue in ballast tanks (Penkala *et al.,* 2004). Acrolein® is claimed to react with water and particulate matter within the ballast tanks resulting in a discharge concentration of zero ppm, thus allowing its safe discharge over board. Laboratory studies conducted by Penkala *et al.* (2004) determined that the product has a half-life of 20-25 h for concentrations of 1, 5 and 10 ppm when prepared in natural port water; however, results from the shipboard trials found that the rate of degradation was much faster. For example, tanks that were treated with 9 ppm had substantially decreased concentrations of <2.5 ppm within 24 h (Penkala *et al.,* 2004) suggesting that the physical and chemical conditions inside the ballast tank increase the degradability of Acrolein®. Acrolein® may also be deactivated by sodium sulphite before discharge, however this would act to increase the treatment cost. The current estimated cost of Acrolein® for treating ballast water is between US$0.16 and 0.19 per tonne. Future research should assess 1) the ability of Acrolein® to inactivate resistant ballast water organisms such as dinoflagellate cysts; and 2) the capacity of the product to inactivate organisms in ballast tank sediment.

Hydroxyl radical treatment

One of the more recent treatment options proposed for the control of ballast water organisms involves the onboard generation of hydroxyl- and oxygen radicals. These free radicals are aggressive and can break down almost any organic compound to carbon dioxide and water (Taylor *et al.,* 2002). It is recommended as a tool for controlling ballast water organisms during ballasting and/or deballasting. The

hydroxyl radicals are predominantly produced from the positive ions O_2^+ and N_2^+ reacting with water, and the concentration required to kill microorganisms is reportedly only 0.63 mg/L (Bai *et al.,* 2005). At this concentration, unicellular algae, protozoans and bacteria are killed within 2.67-8 seconds (Bai *et al.,* 2005; Zhang *et al.,* 2005). The main reasons of cells death are lipid peroxidation, destruction of cell DNA and RNA, and damage to the antioxidant enzyme system (Zhang *et al.,* 2006). No efficacy data exist for resistant life stages such as cysts and spores.

This method also has the following advantages: 1) ballast water quality is improved, as turbidity has shown to be decreased to 50% following the 2.67s treatment; 2) the radicals are reported to be short-lived (nanoseconds), decomposing into water, oxygen and carbon dioxide, making it an environmentally sound technique; and 3) the equipment is small in size, operationally simple and cost effective (Bai *et al.,* 2005). Bai *et al.* (2005) implies that the running cost of hydroxyl radical treatment is $1/30^{th}$ the cost of ballast water exchange; however the initial cost and power requirements would be expected to be considerable. Taylor *et al.* (2002) indicates that corrosion problems maybe a concern, unless the reactor is kept well separated from the main ballast piping system, however this technology should still be pursued for ballast water applications, as it may provide a cost effective, safe, environmentally friendly ballast water treatment. Several companies have developed treatment systems that use a combination of mechanical separation and free radical generation. The effectiveness of several of these systems is discussed later.

De-oxygenation

De-oxygenation has been suggested to be a cost effective technique to prevent aquatic introductions while reducing ship corrosion. De-oxygenation can be achieved by the addition of nutrients, glucose or a reducing agent such as a sulphide, by the use of a vacuum chamber, or oxygen can be purged out of the ballast tanks with a continual flow of an inert gas from an onboard generator (Mountfort *et al.,* 1999a; Tamburri *et al.,* 2002, Browning Jr. *et al.,* 2004; Tamburri *et al.* 2004, McCollin *et al.,* 2007b).

Initial results indicated that the addition of glucose or sulphide had minimal effects on living organisms, however the other techniques have been shown to effectively kill

many zooplankton species (including larval stages) and aerobic bacteria but results against phytoplankton have been inconclusive (Table 8). This approach is considered unlikely to remove taxa adapted to low oxygen environments or with resistant stages such as cysts and spores. For example, Anderson *et al.* (1987) reported that the viability of dinoflagellate cysts of *Alexandrium tamarense* was not reduced when stored in an oxygen-deprived environment.

Mountfort *et al.* (1999a) tested the effectiveness of de-oxygenation by the addition of glucose or sulphide. The addition of glucose did not significantly reduce the oxygen concentration and was ineffective in killing *Coscinasterias calamaria* starfish larvae or *Undaria pinnatifida* seaweed zoospores over a 28 day period, and sulphide addition was capable of eliminating *C. calamaria* larvae but it did not completely kill *U. pinnatifida* zoospores (Mountfort *et al.*, 1999a). Conversely, studies conducted by McCollin *et al.* (2007b) found that the addition of nutrients to ballast water stimulated the growth of bacteria resulting in an anoxic environment, which caused a reduction in the abundance and viability of zooplankton but the results for phytoplankton showed no effect. Similarly, experimental trials using vacuum chambers to suffocate marine organisms have been shown to remove dissolved oxygen from ballast water to levels below 1 ppm resulting in the elimination of many zooplankton species (Browning Jr. *et al.*, 2004) but the effect on phytoplankton was not clear.

Recently, preliminary experiments indicate that the combination of low oxygen (2%) and high carbon dioxide (12%) is capable of eliminating in excess of 95% of zooplankton and invertebrates within several hours, and can kill >99% of *Vibrio cholerae* in 24 h, however experimental data is unavailable on the effects on phytoplankton, cysts and spores (Husain *et al.*, 2004). Cost estimates for this type of treatment system range from US$135,000 to in excess of US$3 million depending on ballast capacity and operating cost are approximately US$0.06 per tonne of ballast water (Lloyd's Register, 2007).

NEI Treatment Systems, LLC of Los Angeles, California has developed the Venturi Oxygen Stripping™ system- de-oxygenation technology that rapidly removes 95% of dissolved oxygen from ballast tanks. The system removes oxygen from ballast water by the introduction of an inert gas into the water as it is being pumped into ballast

tanks. Initial laboratory experiments conducted under a range of environmental conditions demonstrated that dissolved oxygen levels dropped to 0.27-0.87 ppm leading to greater than 99% mortality of zooplankton (copepods, barnacle larvae, polychaete larvae, cladocerans, crustacean nauplii, bivalve larvae and nematodes) in less than 48 h, however, the system was less effective against phytoplankton and no obvious difference in bacterial abundances were observed (Tamburri *et al.,* 2004). Further testing at the Chesapeake Biological Laboratory showed promising results. The system was capable of eliminating 100% of zooplankton and phytoplankton after 120 h of treatment, and reduced the levels of *Enterococci* and *E. coli* bacteria by more than 99.9% (NEI Marine, 2007). In addition, biological trials from a shipboard installation onboard the bulk carrier the *TECO Ocean* suggest that the treatment system is capable of meeting the D-2 discharge standard set by the IMO when operating at a flow rate of $1000m^3/h$, however experimental data are not available. The cost of the Venturi Oxygen Stripping™ system ranges from US$150,000 to $400,000 depending on the flow capacity of the ship and operating costs are approximately US$0.05 per tonne of ballast water (Lloyd's Register, 2007).

The above results indicate that de-oxygenation may eliminate zooplankton but it is unlikely to be an effective ballast water treatment, as a many organisms such as seaweeds, phytoplankton, cysts and spores, anaerobic bacteria and many viruses are likely to survive such conditions. Although several authors indicate that using de-oxygenation techniques may provide ship owners with a significant economic saving (approximately US$80,000-100,000 per year) due to a reduction in ballast tank corrosion (Deacutis and Ribb, 2002; Browning Jr. *et al.,* 2004), it is suggested that alternating back and forth from anoxic conditions to air as well as the stimulation of anaerobic bacteria may act to enhance corrosion rates (Oemcke, 1999; Tamburri *et al.,* 2004).

pH adjustment

Many organisms cannot survive large variations in pH (Muntisov *et al.,* 1993). Raising or lowering pH level in ballast tanks can be achieved by the addition of alkali or basic chemicals and may effectively destroy many organisms. However, this technique has several drawbacks. Firstly, lowering the pH may have significant

effects on corrosion, while elevating the pH may result in chemically unstable water (Oemcke, 1999). Secondly, altering the pH involves the addition of chemicals to ballast tanks by crew members, consequently requiring a large storage space and increasing safety risks. Thirdly, this treatment would result in production of vast quantities of residues that would need to be kept onboard and disposed of safely in landfills, and ballast water pH may need to be restored prior to discharge by the addition of neutralising agents (Muntisov *et al.,* 1993). Finally, pH adjustment may not render all ballast water organisms inactive, especially cysts, spores and other resistant physiological resting stages. For example, Bolch and Hallegraeff (1993) showed that the germination of dinoflagellate cysts of *G. catenatum* was not affected after 24 h exposures to pH values ranging from 2 to 10 and Kurizian *et al.* (2001) found no difference in mortality of mixed zooplankton species between control and elevated pH (8.5 to 10) samples. Bacteria are also capable of surviving wide variations in pH. The optimal pH for rapid multiplication of *V. cholera* on copepods is around 8-9, with a decline in multiplication evident at an acidic pH (6-6.5) (Huq *et al.,* 1984). However, this decline in abundance may be explained by the death of the copepods, rather than the mortality of the bacterium.

Salinity adjustment

Salinity adjustment is aimed to inactivate or destroy marine organisms present in ballast water by increasing or decreasing the salinity of the water, as it is believed that freshwater organisms cannot survive oceanic and estuarine conditions. Conversely, survival of oceanic and estuarine species is likely to be reduced when challenged by freshwater conditions. As not all ships will travel between freshwater and oceanic conditions this procedure could be achieved by the addition of salts to ballast tanks or by the use of an onboard de-salinisation unit. The latter is considered to be expensive, extremely time consuming and would require an enormous amount of energy.

Another factor limiting the use of salinity adjustment for the treatment of ballast water is that many pathogens and resting stages of organisms are likely to survive such treatments. For example, the survival and viability of non-culturable estuarine *E. coli* and *V. cholerae* was unaffected over a range of salinities from 5 to 33 g/L (Xu

et al., 1982; Huq *et al.,* 1984; Munro and Cowell, 1996). Similarly, *G. catenatum* cysts exposed to freshwater and salinities between 15 and 50 g/L were unaffected; however, extreme salinities of 100 g/L prevented their successful germination (Bolch and Hallegraeff, 1993). Salinities in this extreme range are not considered economically or practically achievable in onboard situations, therefore excluding the use of salinity adjustment as a ballast water treatment option.

6.5 Multi-Component Treatment Systems

Many ballast water treatment systems use a combination of treatment options (Table 9). A number of these systems that are currently under commercial development include:

Mechanical treatment and ozone disinfection

Mitsui Engineering and Shipbuilding Co., Ltd in conjunction with the Japanese Association of Marine Safety have developed the Special Pipe Ballast Water Management System (combined with ozone treatment). The system consists of four treatment processes; a pre-treatment unit, the disinfection unit, a gas/liquid separation unit and discharge unit. The disinfection unit terminates organisms using sheer stress and cavitation produced by the special plate structures of the 'Special Pipe' and the ozone treatment enhances the killing effect. The pre-treatment unit prevents the blockage of the disinfection unit, the gas/liquid separation unit prevents gaseous ozone entering the ballast tanks and the discharge unit decomposes any remaining oxidant in the ballast water to safe-guard against chemical discharge during de-ballasting. The system has recently been granted basic approval from the IMO for the use of active substances (GESAMP, 2008).

Land-based tests of a prototype system using a flow rate of 20 m^3/h showed that a single passage through the pipe resulted in the termination of 54.8% of all phytoplankton and of 65.1% of zooplankton and mortality was increased to 99 and 89%, respectively, by injecting 1mg/L of ozone into the seawater (Kikuchi *et al.,* 2004). Further experimentation on an up-scaled model found that the system was capable of eliminating 69.6% of phytoplankton and 94.3 % of zooplankton after a

single passage through the system without the addition of ozone when operating at a flow rate of 115 m^3/h and mortality was increased to 80% and 100% of phytoplankton and zooplankton, respectively, by a two-time passage through the system (Kikuchi *et al.*, 2004). Furthermore, 85% of phytoplankton and 100% of zooplankton were eliminated at a flow rate of 150 m^3/h following a single passage through the system (Kikuchi *et al.*, 2004). This indicates that the effectiveness of the 'Special Pipe' component of the treatment system is increased with an increase in flow rate. Efficacy data on the removal of bacteria are not available; however, the secondary ozone treatment is likely to inhibit bacterial growth if TRO concentrations are maintained at around 1 ppm. Advantages of the system are that it is easily retro-fitted to existing ships and it does not require the storage of large amounts of chemicals onboard. Disadvantages include potential health and safety issues for crew and possible increased corrosion of ballast tanks due to the application of ozone, and it may not be economically feasible for ships with high ballast pump flow rates. The system costs approximately US$ 1 million for installation on an existing container ship with an estimated operating cost of US$0.15 per tonnes of ballast water (MEPC, 2006).

Filtration, cavitation, electrochemical disinfection and de-oxygenation

The Oceansaver® is a Norwegian-made multi-component treatment system consisting of a mechanical filtration unit, a hydrodynamic cavitation chamber, an electrochemical disinfection unit and a nitrogen super-saturation generator. The mechanical filtration unit used is an automatic self-cleaning 50 μm wedge wire filter that operates during ballast intake returning trapped organisms and sediment back to the source location. Efficiency tests have shown that the filter effectively removes only 50-70% of material over 50 μm (Andersen, 2007)

The hydrodynamic cavitation unit involves the formation and implosion of cavitation bubbles which generate forces and shockwaves that affect particles and organisms larger than 10 μm. Land-based experimental testing has shown that the cavitation unit allows the other treatment components in the system to act more effectively (MEPC, 2007e).

The electrochemical disinfection unit produces active substances electrochemically from seawater *in-situ* in a side stream of the main ballast water without the addition of any chemical to the water. The active substance formed is primarily hypochlorous acid with small concentrations of chlorine gas, ozone, hydrogen peroxide, chlorine dioxide and hypochlorite. The treated water is then re-injected back into the ballast water. This component of the system would not be effective when operating in freshwater; however, it may be possible to generate active substances using an external source of brine or seawater (MEPC, 2007e).

The nitrogen super-saturation unit injects nitrogen into the ballast water to reduce the dissolved oxygen concentration to levels of 2-3 mg/L (Andersen, 2007). This leads to hypoxic conditions in the ballast water preventing the re-growth of organisms that require oxygen for their survival. The treated water is then aerated during ballast discharge to avoid the discharge of hypoxic water.

The system has been in operation on several vessels including the MV *Federal Welland* and *Hual Trooper* since 2005 but experimental data are not available. One advantage of this system is that it may be run in several configurations depending on the level of treatment required. For example, following the primary filtration step, any combination of the three secondary treatments may be used depending on the particular properties of the ballast water.

Disadvantages include potential human-health impacts on crew members due to the production of active substances, including chlorine gas and carcinogenic trihalomethanes (THM's). Initial acute toxicity tests carried out following the electrochemical treatment step found that samples taken immediately after treatment and 5 days after treatment showed aquatic toxicity (MEPC, 2008b). Although no total residual oxidants were detectable after 24 h, measurements of the formation of disinfection by-products and other chemicals showed a significant increase in the level of several THM products (trichloromethane/bromoform, dibromochloromethane and tribromomethane) directly after treatment and after 5 days of storage (MEPC, 2008b). These disinfection by-products have been shown to persist and accumulate in the marine environment and raise human genotoxicity/carcinogenicity concerns.

Another issue that require careful consideration includes potential increases in ballast tank corrosion.

It is suggested that the Oceansaver® system is capable of treating in excess of 10,000 m^3 of ballast water per hour, however several treatment systems will be required to operate at this level. The technology is expected to cost approximately US$800,000 per unit with an operating cost of $0.06 per tonne of ballast water (Lloyd's Register, 2007). The system has received basic approval from the IMO for its use of active substances but further detailed investigations of potential environmental and human-health risks are required before final approval can be granted (MEPC, 2008b)

Cyclonic separation, filtration and chemical biocide treatment

Hamann AG and Degussa AG of Germany have produced the SEDNA® (Safe, Effective Deactivation of Non-indigenous Aliens) - a modular ballast water treatment system which consists of a two-step physical separation and a secondary biocide treatment that operates during ballast intake only (Fig. 2). Physical separation is conducted by a number of hydrocyclone devices and a self-cleaning 50 micron filter that function to reduce the sediment load of the ballast water and remove organisms greater than 50 μm. These devices increase the stress on organisms present in the ballast water, resulting in physical damage to the organisms as well as an increased sensitivity towards the secondary chemical treatment (HSB, 2006). The secondary treatment uses the oxidative biocide Peraclean® Ocean to provide complete disinfection of small organisms (<50 μm) and bacteria. The manufacturers suggests that the SEDNA® system is superior to other ballast water treatment systems as it is not limited with regard to water sediment load, salinity and temperature or voyage length; it is fully automated and maintenance can be carried out by trained ship personnel; it can be customised for ballast pump capacities ranging from 50 to 2000 m^3/h; and the chemical is fully biodegradable and only requires a retention time of 24 h in ballast tanks before it can be safely discharged overboard (HSB, 2006; Lloyd's Register, 2007).

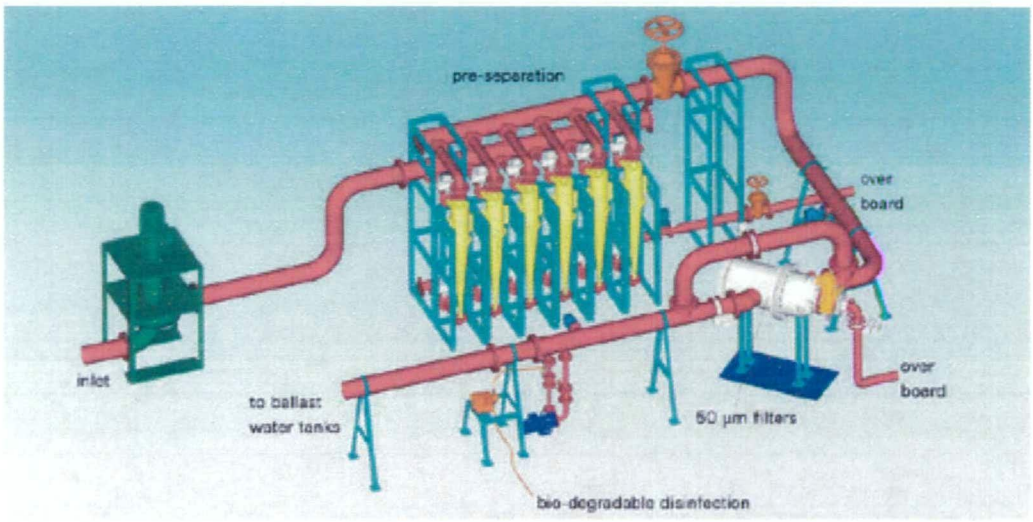

Fig. 2. Schematic diagram of the three treatment stages of the SEDNA® ballast water treatment system (http://www.hsbinternational.nl).

Results of sediment removal efficacy tests conducted by the manufacturer suggest that the system is capable of removing up to 40% of particles >10 µm and >95% of the suspended solids >30 µm and biological efficacy tests have shown that the system is capable of exceeding the D-2 standard of the IMO Ballast Water Convention (HSB, 2006). Independent tests carried out by Veldhuis *et al.* (2006) on a SEDNA® system capable of treating 530 m^3/h found that the physical separation components of the treatment system did not remove the sediment component of the natural harbour water, which consisted of mainly clay (<2 µm) and silt (<63 µm) particles, or *Phaeocystis globosa* cells but did reduce the abundance of some zooplankton species. Although the hydrocyclone did not effectively reduce the total suspended solids of the incoming water, it did function to alter the structure of gelatinous phytoplankton colonies of *P. globosa* in a manner that the self cleaning filters were not clogged as often. For example, when the hydrocyclone devices were not operating, the filter was clogged within 10 min, whereas under full operation the filter self-cleaned every 60 to 90 min (Veldhuis *et al.*, 2006). Lastly, the Peraclean® Ocean disinfection step effectively eliminated all zooplankton species, all phytoplankton species examined (*Chaetoceros muellerii, Chlorella autotrophica, P. globosa*) as well as bacteria at a concentration of 150 ppm (Veldhuis *et al.*, 2006). At this concentration (150 ppm), bacterial regrowth was inhibited for a period of 6-10 days indicating that Peraclean®

Ocean did not achieve a full disinfection of the incoming water (Veldhuis *et al.*, 2006).

Potential disadvantages of the system include the cost effectiveness of Peraclean® Ocean (approximately US$0.30 per tonne of ballast water), possible increased corrosion due to the use of an oxidative biocide, the reduced effectiveness of Peraclean® Ocean against resistant organisms and in the presence of sediments, health and safety issues and space requirements relating to the need to store large amounts of noxious chemicals onboard and possible residual toxicity of treated ballast water, as several studies have found that the biocide degrades at a much slower rate than claimed by the manufacturer (e.g. Gregg and Hallegraeff, 2007; de Lafontaine *et al.*, 2008a).

Nonetheless, the SEDNA® ballast water treatment system using Peraclean® Ocean is the first operational system that has received both Basic and Final Approval by the IMO for its use of an active substance as well as for the whole system and Type Approval by the relevant national regulatory authority, the Federal Maritime and Hydrographic Agency, Berlin (MEPC, 2007d).

Ultrasound and filtration

Environmental Technologies Inc. and Qwater Corporation of the United States are developing treatment systems that combine filtration and ultrasound. Although, no efficacy data are available for either system, it is believed that pre-filtration may decrease the effectiveness of ultrasound treatment by removing particulate matter that acts to kill smaller organisms through collisions. An alternative approach that deserves investigation is the combination of ultrasound with chemical biocides and/or UV treatment. Mason *et al.* (2003) and Gavand *et al.* (2007) have demonstrated that the efficacy of ultrasound is increased by the addition of chemicals including chlorine, ozone and hydrogen peroxide; and similarly, Sassi *et al.* (2005) showed that the efficacy of ultrasound treatment can be significantly improved when combined with UV irradiation. At the current stage of development, however, ultrasound technologies would not be considered appropriate for the shipboard treatment of ballast water due to high capital and operating costs and high power requirements.

Mechanical separation and electrochlorination

Several companies have produced two-stage ballast water treatment systems that combine primary separation devices followed by disinfection by electrochlorination. Greenship Ltd of the Netherlands has designed a shipboard ballast water treatment system that includes the 'Sedimentor'- a hydrocyclone for removing sediment and biota during uptake, and the 'Terminox'- an electrolytic cell that produces sodium hypochlorite for disinfection (Fig. 3).

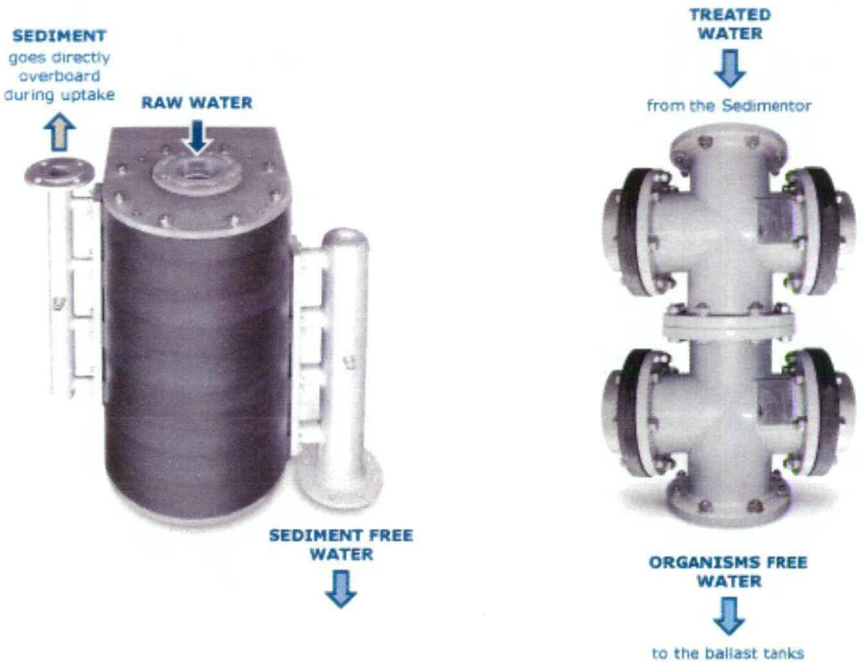

Fig. 3. Main components of the Greenship ballast water treatment system. (A) the 'Sedimentor'- a hydrocyclone device; and (B) the 'Terminox'- a sodium hypochlorite generator (http://www.hme.nl).

Efficacy experiments conducted on a laboratory test unit with a capacity of 50 m^3/h suggest that the 'Sedimentor' is capable of removing 100% of particles 20 μm and larger, and 80% of the particles greater than 10 μm (MEPC, 2007a). This would act to remove most phytoplankton and zooplankton species from the ballast water and would significantly minimise the sediment load increasing the killing effect of the sodium hypochlorite. Tests on the effect of the electrolytic cell showed a killing efficacy of >99.99% of heterotrophic bacteria (including *E. coli*) after 2 min when performed with 10 ppm sodium chlorite (MEPC, 2007a); indicating that the system is capable of meeting the bacterial component of the IMO discharge standard. In addition to the laboratory test, supplemental land-based tests were conducted on

natural seawater using a system with a capacity of 100 m^3/h. Results showed that the system removed 100% of organisms ≥50 μm, 98-100% of organisms 10-50 μm and eliminated 99.9-100% of aerobic heterotrophic bacteria including *E. coli* and Enterococcae (MEPC, 2007a). For best results a free chlorine concentration of at least 2.6 ppm is recommended. The system is capable of producing higher levels of chlorine; however concentrations above 3 ppm should not be used as they pose environmental problems due to the formation of toxic by-products. Residual measurements of sodium hypochlorite found that no trace of free chlorine was found in treated ballast water after 90 min, however, several trihalomethane and haloacetic acid products were detected (MEPC, 2007a). Other drawbacks of the system include potential increases in ballast tank corrosion and concerns regarding the toxicity of treated ballast water at discharge. Toxicity tests have shown that treated ballast water caused no negative effects on larvae of the brine shrimp (*Artemia franciscana*) or freshly fertilised eggs and yolk-sac larvae of the sole (*Solea solea*) after 24 h, but the diatom *Skeletonema costatum* did not survive exposure to treated ballast water even in tests conducted 96 h after treatment (MEPC, 2007a). Preliminary shipboard testing has shown that the system operates effectively in salinities of >3 ‰ meaning that the system can operate in 96% of all situations worldwide (MEPC, 2007a). In order to operate in freshwater, seawater or brine needs to be injected into the system. The estimated cost for full installation of Greenship's Ballast Water Management System is US$2,300,000 for a system capable of treating a flow of 2000 m^3/h (Carney *et al.*, 2008). Operational costs for this system are not available.

Severn Trent De Nora builds a similar treatment system, the BalPure® Ballast Water Treatment System, but this differs in that filtration is used prior to electrochlorination. The capital cost of this system is considerably less than the Greenship system ($US500,000 for 2000 m^3/h flow capacity) and has an operational cost of US$0.02 per tonne of ballast water (Lloyd's Register, 2007).

Filtration and free radical treatment

RWO GmbH Marine Water Technology and Veolia Water Solutions and Technologies have developed 'CleanBallast!'- a two-stage ballast water treatment system that consists of a mechanical separation step and an electrochemical treatment

step (Fig. 4). The mechanical filtration step uses self-cleaning disk filters to remove suspended solids, sediment and larger organisms and prevent the accumulation of sediment in ballast tanks. The filtration system consists of an array filter housings that operate in parallel and are self-cleaned one at a time allowing filtration to operate without reducing the ballast water flow rate. The electrochemical treatment step produces active substances *in-situ* by the Ectosys® electrochemical treatment cell. This unit produces several different disinfectants (free hydroxyl- and oxygen radicals and a small amount of hypochlorous acid) directly from seawater. The treatment system operates during ballast intake, however land-based tests showed that re-growth of organisms does sometimes occur, therefore, during ballast discharge the filter unit is by-passed and the ballast water is treated again with the electrochemical treatment cell (MEPC, 2007c).

Only a very limited amount of efficacy data is available on the 'CleanBallast!' system. The filter component has been shown to remove 100% of *Artemia salina* cysts (NAG Marine, 2007) and biological efficacy tests conducted in river, brackish and seawater at four different locations indicate the system is capable of exceeding the D-2 standard of the IMO Ballast Water Convention for the three size classes of organisms (MEPC, 2006), however experimental data are not presented.

Fig. 4. The RWO GmbH Marine Water Technology 'CleanBallast!' ballast water treatment system (http://www.nagmarine.com).

Two advantages Ectosys® electrolysis has over conventional electrolytic chlorine generators is that it can operate effectively in freshwater without the need to add brine or seawater and produces less chlorine and therefore less chlorine disinfection by-products. Results from acute toxicity studies on treated effluent have shown no significant effects caused by treatment with the 'CleanBallast!' system. The system does produce small amounts of hydrogen and chlorine gas but these are not regarded as a problem if the system is adequately vented during operation. Low concentrations of several THM and HAA by-products as well as bromate are also formed, especially in waters with high salinity (MEPC, 2007c). No corrosion data are available for the 'CleanBallast!' system. The system has a capacity of 500 m^3/h and due to its modular design can be adapted to all flow rates by the joining of numerous units. The price for a system is not available, though operating costs are only due to the power consumption of the unit.

Alfa Laval Tumba AB of Norway in partnership with global shipping firm Wallenius has also developed a chemical-free treatment system that uses filtration and chemical disinfection with free radicals produced in the patented AOT (Advanced Oxidation Technology) unit. During ballasting, water passes through a 50 μm filter to remove any large particles and organisms. Water then continues to the AOT unit, which

contains titanium dioxide catalysts that generate free radicals when hit by UV light. During deballasting, water is passed again through the AOT unit to eliminate any organisms that may have re-grown during transit. The treatment system uses no chemicals or additives and has no toxic residuals. Laboratory pilot-scale tests conducted at a flow rate of 25 m^3/h have shown that 'PureBallast' system eliminates over 99.999% of organisms $\geq$10 μm and can achieve a 99.999% reduction in the concentration of *E. coli* bacteria (Alfa Larval, 2008). The system has undergone full-scale testing onboard the Wallenius car carrier, the MV *Don Quijote,* but efficacy results are not available. Wallenius also plans to install 'PureBallast' on all newly built car carrier vessels. The 'PureBallast' system has received full approval from the IMO for the use of active substances and has recently been granted full Ballast Water Type Approval from the relevant Norwegian authorities.

Due to the modular design, the system can be adapted for different vessel types and can cover a flow range of 25-5000 m^3/h. The operational cost of the system is approximately US$0.06 per tonnes of ballast water (MEPC, 2006). Capital costs are not available.

Table 9. Summary of treatment processes and key system data (capacity, footprint, active substance approval status, costs) of several multi-component ballast water treatment technologies currently under commercial development (modified from Lloyd's Register (2007) and Carney *et al.* (2008))

Manufacturer	System Name	Treatment Process	Capacity ('000's m^3/h)	Footprint (m^3)		Active Substance Approval (if applicable)		Efficacy	Capital Cost ($US)		Operating Cost (US$ per tonne)
				200 m^3/h	2,000 m^3/h	Basic	Final		200 m^3/h	2000 m^3/h	
Alfa Laval Tumba AB	Pureballast	Filtration + advanced oxidation	5	3	10	Yes	Yes	99.99999% *E. coli* 99.999% organisms > 10 μm	NA	NA	0.06
Electrichlor Hypochlorite Generators Inc.	Electrichlor	Filtration + electrochlorination	>10	3	NA	No	No	NA	NA	NA	0.019
Environmental Technologies Inc.	-	Filtration + ultrasound	>10	NA	6	Not applicable	Not applicable	NA	NA	500,000	0.005
Greenship Ltd	Greenship	Cyclonic separation + electochlorination	>10	1.6	15	Yes	No	99-100% heterotrophic bacteria 100% organisms >50 μm 98-100% organisms 10-50 μm	300,000	2,300,000	NA
Hamann AG/ Degussa AG	SEDNA®	Cyclonic separation + filtration + chemical biocide	2	4.3	36	Yes	Yes	Exceeded D2 standard 100% selected phytoplankton and zooplankton	NA	NA	0.3
HITACHI	Clearballast	Flocculation + magnetic separation + filtration	>10	20	100	Yes	No	100% organisms > 10 μm >99.8% *E. coli* Exceeded D2 standard	NA	NA	NA
Hyde Marine Inc	-	Filtration + UV	>10	7	35	Not applicable	Not applicable	87 % organisms 100% coliform bacteria	NA	NA	NA
Marenco Technology Group	Marenco	Filtration + UV	1	2	NA	Not applicable	Not applicable	NA	135,000	NA	0.1
Mitsui Engineering and Shipbuilding Co., Ltd	Special Pipe	Mechanical treatment + ozone (1 ppm)	NA	13	NA	Yes	No	99% phytoplankton 89% zooplankton	NA	1,000,000	0.15
Oceansaver AS	Oceansaver®	Filtration + cavitation electrolysis + de-oxygenation	>10	NA	NA	Yes	No	NA	NA	NA	NA
Optimarin	Optimarin	Filtration + UV	>10	variable	variable	Not applicable	Not applicable	NA	NA	500000	NA
Qwater	Qwater	Filtration + ultrasound	NA	15	30	Not applicable	Not applicable	NA	NA	NA	NA
Resource Ballast Technology	-	Filtration + electrochlorination + cavitation	>10	2	4	Yes	No	NA	150000	250000	NA
RWO GmbH Marine Water Technology/Veolia Water Solutions	CleanBallast!	Filtration + electrochemical treatment	>10	3	24	Yes	No	Filtration unit removed 100% of *Artemia* cysts Total system exceeded D2 standard	NA	NA	NA
Severn Trent De Nora	Balpure®	Filtration + electrochlorination	>10	11	11	Yes	No	NA	350,000	500,000	0.02

6.6 The 'ballast-free ship' concept

Another technology that could reduce the ballast-mediated transfer of non-indigenous organisms is the 'ballast-free ship' concept. This concept was patented in 2004 and is intended for new-vessel construction only. It involves redesigning the ballast system of ships so that a constant flow of water runs through the entire length of the ship essentially eliminating the transport of ballast water (Fig. 4). The concept involves replacing traditional ballast tanks with longitudinal, structural ballast trunks that extend beneath the cargo region of the ship below the ballast draft (Parsons and Kotonis, 2007). These trunks are flooded with seawater to reduce the buoyancy of the vessel and due to the motion of the ship through the water, a slow, continuous flow of 'local seawater' moves through these open ballast trunks (Parsons and Kotonis, 2007). When a ship is required to take on cargo, the ballast trunks can be isolated from the ocean by valves, then the water is pumped out using conventional ballast pumps.

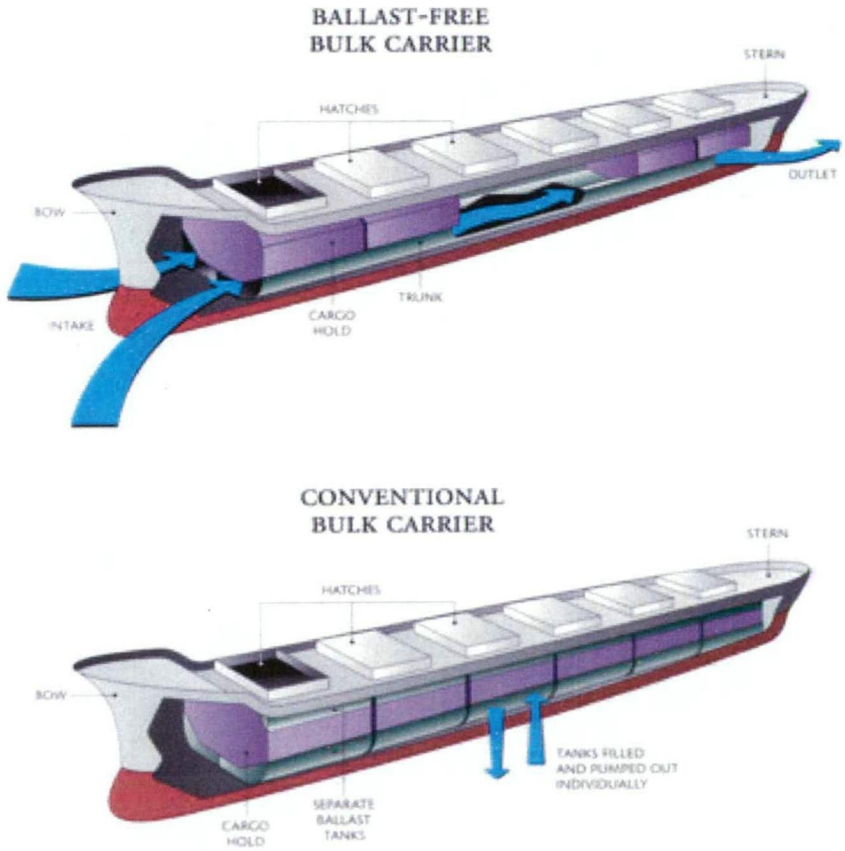

Fig. 4. Operating principle of the 'ballast-free ship' concept compared to traditional ballast tanks (http://www/umich.edu).

Computer modelling and scale model tests have shown that the idea is technically and economically feasible (Parsons and Kotonis, 2006, 2007), however the entire vessel design needs to be redeveloped to support the proposed concept and it may only be suitable for certain ship types. If successful, this design would eliminate the need for costly ballast water treatment equipment or chemicals and is even suggested to result in as much as a 7.3% reduction in the power needed to propel the ship (Erickson, 2008). The researchers suggest that the new design would result in a net capital-cost saving of around US$540,000 per ship and combined with the expected fuel savings, total transport costs would be cut by $US2.55 per tonne of cargo (Erickson, 2008).

6.7 Organism viability testing for full-scale ballast water treatment systems

Accurate sampling and viability testing in onboard situations is a significant challenge as standardised laboratory methods are not applicable to the changing conditions in ships' ballast water operations. Quantifying the number of viable organisms greater than 50 μm is particularly difficult as several tonnes of water must be sampled. Gollasch (2006) illustrates one example of a specially designed device for sampling ballast water organisms greater than 50 μm in dimension. The device consists of a flow meter, non-stick sample bag and cod end with removable filter panels. Onboard tests have proven that the device is capable of efficiently sampling more than 2,500 litres of ballast water in less than 30 min (Gollasch, 2006).

Efficacy testing also requires a scientific analysis of organism viability as the D-2 discharge standard refers to live organisms. Several methods are available for the assessment of organism viability for full scale ballast water treatment systems. Organisms may be assessed microscopically for morphological changes or signs of movement, however this technique is not appropriate for many species, such as diatoms and resting stages, it may not be accurate in shipboard studies due to the motion of the ship and it requires all individual organisms to be examined making it a very time consuming approach.

An alternative approach involves the use of vital stains in conjunction with fluorescent microscopy or flow cytometry. Gollasch *et al.* (2007) highlights two different methods that are available to rapidly determine organism viability. One method uses nucleic acid stains, such as Sytox® Green, that are able to penetrate the compromised plasma

membranes of dead cells but will not cross the membranes of live cells. The stained nucleic acids of dead cells fluoresce bright green when excited by a 450-490 nm light source. This technique has been successfully used to assess viability in a range of organisms including bacterial cells (Roth *et al.*, 1997; Lebaron, *et al.*, 1998), a wide range of vegetative marine microalgal species (Veldhuis *et al.*, 1997; Veldhuis *et al.*, 2006), dinoflagellate cysts (Binet and Stauber, 2006), the copepod *Calanus helgolandicus* (Buttino *et al.*, 2004), and the nematode worm *Caenorhabditis elegans* (Gill *et al.*, 2003).

The other method stains the cell membrane or tissue of living cells. This can be achieved using visual stains such as Neutral Red or fluorescent stains such as FDA (Fluoroscein diacetate) or DAPI (4', 6-diamidino-2-phenylindole). The latter fluorescent stains may be used in combination with nucleic acid stains allowing for two-colour visualisation of live and dead cells. One limitation of vital stains is that neither of the methods works for all types of species within the two size groups specified in the D-2 discharge standard (Gollasch *et al.*, 2007).

Another emerging technology for the assessment of organism viability in ballast water samples is the FlowCAM®. The FlowCAM® is a continuous imaging flow cytometer that combines microscopy, flow cytometry, imaging and fluorescence technologies allowing for rapid visual and analytical comparison of untreated to treated ballast water. It can detect organisms from 0.5 μm to 3 mm and can quantify and image phytoplankton and zooplankton 3 μm to 3 mm in size and has the ability to detect auto-fluorescence, stain-induced fluorescence or visual stains enabling the system to differentiate live versus dead organisms. It is a portable system and is even available as a submersible system that could be lowered into ballast tanks. This technology has been used successfully to determine viability in a range of cultured organisms including rotifers (*Brachionus plicatilis*), *Artemia* and dinoflagellates (*Heterocapsa*) stained with FDA and Sytox® Green, and has been used to determine zooplankton viability using Neutral Red staining in natural harbour samples (Peterson, 2008; Poulton, 2008). No data are available regarding the accuracy of the FlowCAM® in shipboard ballast water treatment viability analysis.

6.8 Conclusions and recommendations

The variable efficacy and operational limitations of ballast water exchange have led to significant financial investment in the research and development of ballast water treatment technologies. Each of the shipboard treatment options discussed above have their own advantages and disadvantages with regard to factors such as biological efficacy, cost, ship and crew safety, power and space requirements, operational efficiency and environmental soundness.

Mechanical separation technologies may reduce the concentration of organisms and sediment taken in during ballasting but they are unlikely to satisfy the IMO D-2 discharge standard. These devices may act to improve water clarity thus improving the performance of secondary treatments. Many of the ballast water treatment technologies employ a combination of either filtration and/or cyclonic separation followed by chemical biocide dosing, electrolytic treatment or UV irradiation.

Treatment systems that combine mechanical separation and UV irradiation have the advantage of being environmentally sound and relatively cost effective. These systems are intended for ships with ballast water flow rates up to ~1000 m^3/h. The treatment of larger flow rates is possible, but the systems would consume a prohibitive amount of electricity and may be subject to space limitations. Another disadvantage is that they are unlikely to eliminate all ballast water organisms, as they are not able to deliver a stable lethal dose across a wide range of water quality conditions and many organisms have been demonstrated to be resistant to UV treatment. In addition, the presence of sediment in ballast water is likely to shield small organisms, such as bacteria, from UV irradiation. Further research should aim to enhance the efficacy of UV treatment by either improving water clarity or by combining with another treatment such as heat, ultrasound or chemical reagents.

Ultrasound treatment is also considered as an environmentally safe option but at the current stage of development, it would not be considered appropriate for the shipboard treatment of ballast water due to high capital and operating costs and high power requirements. This technology is also likely to be limited by the high flow rates of ballast pumps. This problem may be overcome by recirculating ballast water through the treatment device. This approach may also increase the efficacy of UV irradiation

but it may not be possible on short voyages due to time constraints and may cause ship stability problems. Further research is required on the biological efficacy of ultrasound in a wide range of water conditions as well as potential health and safety issues and hull integrity problems due to repeated exposure to ultrasound.

The heating of ballast water using waste heat from ships' engines has been demonstrated to be a practical and cost effective treatment options for eliminating ballast water zooplankton and phytoplankton (including resting stages) but concerns exist that the attainable temperatures will not eliminate bacterial pathogens. Promising research has been conducted on several systems that are able to achieve temperatures capable of eliminating bacteria. One system uses additional heat exchangers to reach temperatures of >55°C for short periods but it is not known whether this temperature can be achieved for the entire ballast capacity. The other treatment system uses microwaves to heat water to temperatures in excess of 100°C but at the present stage of development, high energy requirements and costs would eliminate the use of this technology in shipboard situations. Nonetheless, heat treatment deserves further investigation for ballast water treatment as it is an environmentally sound option that can potentially eliminate all ballast water biota including sediment-dwelling organisms.

De-oxygenation by the addition of glucose or reducing agents are not effective ballast water treatment options, however de-oxygenation technologies that are based on the injection of an inert gas are more promising as they are cost effective and do not impact on the aquatic environment as ballast water is re-oxygenated prior to discharge. Disadvantages of de-oxygenation are that it is unlikely to eliminate ballast water organisms adapted to low oxygen environments, such as resting stages, and the process takes several days to asphyxiate organisms and thus may not be appropriate when the voyage length is short.

The Hitachi 'ClearBallast' system that uses flocculating agents and magnetic separation to remove ballast water organisms is a promising technology but it may be limited by space restrictions as the treatment system is large and the recovered material must be stored onboard and disposed of in landfill.

Biocide dosing systems that use proprietary chemicals such as Acrolein®, Peraclean® Ocean and SeaKleen® would have low capital costs and power requirements but chemical costs are significant. Dosing ballast tanks with chemical biocides requires onboard chemical storage areas and the chemicals would need to be available in ports worldwide. Treatment costs and space requirements can be significantly reduced by using onboard chemical generators. The onboard generation of ozone, chlorine, chlorine dioxide and hydrogen peroxide is possible but the capital cost of these systems is significant and all have biological efficacy, safety, operational and environmental concerns. Most chemical treatment systems use mechanical separation and/or cavitation to enhance the killing effect of the biocides (e.g. SEDNA®, Greenship electrochlorination, 'Special Pipe', Oceansaver®). Several treatment systems use no mechanical pre-treatment ahead of the chemical disinfection (Nutech ozone, Ecochlor chlorine dioxide). For all these treatment systems, it needs to be determined whether resistant organisms that reside in ballast tank sediment can be effectively eliminated. The major concern regarding the use of chemical biocides or active substances is the potential environment impacts from the release of toxic ballast water or disinfection by-products. The treatment systems that produce free hydroxyl radicals by either electrolysis ('Cleanballast!') or advanced oxidation ('PureBallast') would be favourable over the other chemical treatments as they are suggested to produce less or no toxic by-products at ballast discharge and, unlike electrochlorination systems, they can operate in both freshwater and seawater. The major limiting factor for these technologies is high power requirements and it is unlikely that the power available onboard ships will be sufficient for the operation of these systems at high flow rates.

At the time of writing this review 10 candidate treatment systems received IMO basic approval to proceed with large scale land-based and onboard ship testing, 3 treatment systems received final approval and 2 received a type approval certificate by an administration which confirms compliance with the ballast water performance standard of the IMO convention. The still limited production capacity for ballast water treatment systems may also not be sufficient to equip all vessels by 2011 when the D-2 discharge standards are planned to apply to the first group of vessels.

In summary, reducing the risk of ballast mediated invasions represents a significant technological challenge. Ideally, a treatment option that is 100% effective is required.

At the present time, no treatment option or multi-component treatment system has proved to be completely effective as each are limited by one or more factors including cost effectiveness, space and energy requirements, environmental soundness, safety and biological efficacy. Many of these limitations relate to the high flow rates and volumes of water that must be treated. Although many of the treatment options are suggested to be able to meet the D-2 discharge standard, all of them require further research on their biological and operational efficacy and safety under full-scale shipboard conditions, in particular, their ability to inactivate resistant organisms such as dinoflagellate cysts. Accurate ballast water sampling and viability testing in onboard situations is a difficult task and is of particular concern. Several promising devices and methods are currently available or under development to improve the accuracy and efficiency of this task.

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Chapter 7

Experimental assessment of translocation of adult bivalve shellfish as a vector for harmful marine microalgal introductions

7.1 Abstract

This study investigated the potential for adult bivalve shellfish to transfer harmful phytoplankton when translocated to new aquaculture areas. Vegetative microalgal cells of the dinoflagellates *Alexandrium catenella, Cryptoperidiniopsis brodyi, Gymnodinium catenatum, Karenia papilionacea, Kryptoperidinium foliaceum, Pfiesteria shumwayae*, the raphidophytes *Chattonella marina, Heterosigma akashiwo* and sexual resting cysts of *Gymnodinium catenatum* were fed to two species of bivalve molluscs (blue mussel *Mytilus galloprovincialis* and Pacific oyster *Crassostrea gigas*) for a period of 48 hours to determine whether algal cells can pass intact through the digestive tracts to establish viable growing populations. Following exposure to the harmful microalgae, the shellfish were placed in filtered seawater and their faecal material collected after 24 and 48 hours and observed for the presence of intact cells or cysts. Dinoflagellate species were found to be more likely to pass through the gut intact, whereas the two raphidophyte species were the least successful. After 24 h depuration in filtered seawater, no *C. marina* cells were observed to be intact in oyster faecal material and no intact cells of *C. marina, H. akashiwo* were visible in faeces from the blue mussel. No intact *K. papilionacea* cells were observed in oyster or mussel faeces produced after 48 h depuration. Samples of the faecal material were inoculated into nutrient medium to evaluate the ability of the ingested cells to recover into growing populations. *Kryptoperidinium foliceum, P. shumwayae* and *C. brodyi* were the most successful at establishing growing populations with cells able to recover in all trials for both the Pacific oyster and blue mussel. *Alexandrium catenella* cells recovered in 100 and 90 % of the oyster trials and 60 and 40% of the mussel trials following 24 and 48 h depuration, respectively. Only 30% of the *H. akashiwo* oyster trials resulted in growing populations and for *G. catenatum,* 90% of the trials recovered into growing populations following 24 h depuration, but this declined to 40% for the Pacific oyster, and 20% for the blue mussel following 48 h depuration. Intact *Gymnodinium catenatum* cysts were recovered from oyster and mussel faeces after 48 h depuration and 63-65% of cysts germinated into healthy swimming planozygote cells, which was less but comparable to the control (71%). Results demonstrate that bivalve shellfish are a probable vector for the transfer of harmful microalgae into pristine areas. Further work is required to determine an efficient, cost effective and safe treatment method to mitigate this significant risk.

Keywords: Bivalve shellfish, Harmful microalgae, Introductions and transfers

7.2 Introduction

Anthropogenic introductions of non-indigenous species can lead to dramatic alterations of the marine environment. There are several means by which species may become introduced. Ballast water transport and hull-fouling transfer by commercial ships have been suggested to be the primary vectors for aquatic introductions (Minchin and Gollasch, 2002). Other mechanisms include aquaculture, seafood, bait and aquarium enterprises; recreational vessels; seaplanes; marine debris and ocean current movements (Carlton, 1985; Carlton and Geller, 1993; Weigle *et al.*, 2005). Of these non-shipping mechanisms, the aquaculture industry has played a substantial role in the introduction of non-indigenous marine organisms around the globe, particularly with regard to the translocation of shellfish (Minchin and Gollasch, 2002)

Large quantities of shellfish are often transferred from one water body to another to establish new aquaculture fisheries or for outgrowing purposes between spat collecting, nursery and outgrowing aquaculture leases (Wolff and Reise, 2002). As aquaculture is one of the fastest growing sectors of the world food economy (Naylor *et al.*, 2001), the increasing trade of shellfish for culture has ignited concerns for the transfer of aquaculture diseases and non-indigenous species. Live shellfish are known to harbour a variety of organisms and have been documented or suggested to be a possible vector for the introduction of parasites and pathogens, harmful microalgal and macroalgal species, and various invertebrates (Humphrey, 1988; Chew, 1990; Minchin, 1996; Wolff and Reise, 2002; Pate *et al.*, 2005; Mineur *et al.*, 2007).

In many instances, little has been done to avoid the transfer of non-indigenous organisms associated with the introduction and translocation of live shellfish. For example, an examination of oyster spat exported from France to Ireland in 1993 revealed the presence of a wide variety of organisms (Minchin *et al.*, 1993) including 67 phytoplankton species, including dinoflagellate cysts (O' Mahony, 1993). When toxic or nuisance phytoplankton species are transferred to pristine areas, vast impacts on the marine environment, native fisheries, aquaculture operations and human health can occur (Hallegraeff, 1992). The apparent global increase in the frequency, intensity and geographical distribution of harmful algal blooms that has been experienced over the past few decades (Hallegraeff, 1992) therefore, could be in part related to the transfer of shellfish species from one location to another.

Here we assessed the potential for harmful microalgae to be transported via the relocation of bivalve shellfish. Previous researchers have shown some phytoplankton species can remain intact in the digestive tracts of certain molluscs and may recover into viable populations once excreted in the bivalve faeces (Carricker, 1992; Bricelj *et al.*, 1993; Laabir and Gentien, 1999; Hégaret *et al.*, 2008). This work builds on studies by Hégaret *et al.* (2008) who assessed the risk of several harmful algal species being transported associated with seven bivalve species that are commercially important in the USA. To our knowledge, studies of this kind have never been done in Australia. Bivalve species tested were the two most commercially exploited species in South Eastern Australia, the native blue mussel (*Mytilus galloprovincialis*) and the non-indigenous Pacific oyster (*Crassostrea gigas*). Aims of this work were firstly to determine whether microalgal cells of the dinoflagellates *Gymnodinium catenatum, Alexandrium catenella, Kryptoperidinium foliaceum, Karenia papilionacea, Pfiesteria shumwayae* and *Cryptoperidiniopsis brodyi*; and the raphidophytes *Chattonella marina* and *Heterosigma akashiwo* can pass intact through the digestive tracts of the Pacific oyster and blue mussel; and secondly, to assess whether the microalgal cells can recover into viable populations following ingestion by the bivalve species.

7.3 Materials and methods

Microalgal cultures, dinoflagellate cyst production and bivalve preparation

Adult blue mussels (*M. galloprovincialis*) ranging in size from 79-90 mm shell length, and Pacific oysters (*C. gigas*) ranging in size from 81-86 mm shell length, were obtained through Tasea Enterprises Ltd. from shellfish farms at Great Bay, Bruny Island (*M. galloprovincialis*) and Pipeclay Lagoon (*C. gigas*) in South East Tasmania. The two species of bivalve molluscs were fed cultured microalgal species obtained from the microalgal culture collection at the School of Plant Science, University of Tasmania. Microalgal species used were the dinoflagellates *Gymnodinium catenatum, Alexandrium catenella, Kryptoperidinium foliaceum, Karenia papilionacea, Pfiesteria shumwayae* and *Cryptoperidiniopsis brodyi*; and the raphidophytes *Chattonella marina* and *Heterosigma akashiwo*. Table 1 identifies the strain characteristics and isolation details of the microalgal species used.

Table 1. Isolation details and characteristics of microalgal strains used.

Species	Algal group	Cell size (μm)	Culture code	Date of isolation	Source	Isolated by	Status
Alexandrium catenella	Armoured dinoflagellate (PSP)	25-32	ACSH02	N/A	Sydney Harbour (NSW)	S. Norwood	N/A
Chattonella marina	Raphidophyte (fish-killer)	30-70	CMJP01	N/A	Japan	N/A	N/A
Cryptoperidiniopsis brodyi	Unarmoured dinoflagellate	6-11	CBDE2	03/2004	Derwent River (TAS)	T.G. Park	N/A
Gymnodinium catenatum	Unarmoured dinoflagellate (PSP)	27-46	GCDE11	N/A	Derwent River (TAS)	M. de Salas	Unialgal
Gymnodinium catenatum	Unarmoured dinoflagellate (PSP)	27-46	GCTRA01	24/05/2000	Triabunna (TAS)	M. de Salas	Clonal
Heterosigma akashiwo	Raphidophyte (fish-killer)	11-25	HAPR01	N/A	Port River (SA)	J. Marshall	Clonal
Karenia papilionacea	Unarmoured dinoflagellate (fish-killer)	18-32	KPPL01	27/05/2003	Port Lincoln (SA)	M. de Salas	Clonal
Kryptoperidinium foliaceum	Armoured dinoflagellate	24-40	KFCR01	4/05/2005	Chapman River (WA)	M. de salas	Clonal
Pfiesteria shumwayae	Unarmoured dinoflagellate (fish-killer)	8-18	CCMP2089	5/01/2000	North Carolina (USA)	L. Haas	N/A

Vegetative cultures of the dinoflagellates *G. catenatum, A. catenella, K. foliaceum* and *K. papilionacea*; and the raphidophytes *C. marina* and *H. akashiwo* were grown in 5 litre culture flasks containing GSe nutrient medium (28‰ salinity) (Blackburn *et al.*, 1989). The dinoflagellates *P. shumwayae* and *C. brodyi* were grown in 500 ml canted-neck tissue culture flasks containing f/2 medium (15‰ salinity) (Guillard, 1975). All nutrient medium was made with filtered seawater (35‰ salinity) collected from Bruny Island, Tasmania. Seawater was filtered using a 0.2μm membrane filter. Cultures were maintained at 17°C (*G. catenatum, A. catenella, K. foliaceum, K. papilionacea*) and 20°C (*C. marina, H. akashiwo*) under 12h dark/ 12h light. Light was provided at an intensity of 100 μmol quanta $m^{-2}s^{-1}$ by a bank of cool-white fluorescent tubes. *Pfiesteria shumwayae* and *C. brodyi* were grown in the dark at 20°C and were fed 100 ml of prey (*Rhodomonas* sp.) every 5 days.

Gymnodinium catenatum resting cysts were produced by inoculating 2 ml culture suspensions of compatible sexual mating strains (GCDE11 x GCTRA01) into 250 ml screw top beakers containing 190 ml of filtered seawater (28‰ salinity) and 10 ml of GSe nutrient medium. The screw top beakers were incubated at 17°C under 12h dark/ 12h light with a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ and were examined at regular intervals for cyst formation. This procedure produced approximately 1500 cysts per beaker after 4 weeks. Prior to experimentation, cysts were stored at 4°C in the dark to prevent premature germination.

Feeding and depuration experiment

All individuals of the two species of bivalve molluscs were placed in a 200 L tank containing filtered seawater (35‰ salinity) for 48 h at 14°C to allow them to depurate. Aeration was provided by a 4-way aquarium bubbler. Following depuration, 10 individuals of each species of bivalve were then transferred to 10 L tanks and exposed to vegetative cells of the eight different microalgal species for 48 h at concentrations equivalent to a natural bloom: 10^6 cells L^{-1} for *G. catenatum* (e.g. Rodriguez *et al.,* 2005), 10^6 cells L^{-1} for *A. catenella* (e.g. Collos *et al.,* 2004), 10^6 cells L^{-1} for *K. foliaceum* (including temporary cysts) (e.g. Kempton *et al.,* 2002), 10^7 cells L^{-1} for *H. akashiwo* (e.g. Keppler *et al.,* 2005), 10^6 cells L^{-1} for *K. papilionacea* (e.g. Whereat, 2007), 10^6 cells L^{-1} for *C. marina,* and 10^4 cells L^{-1} for *P. shumwayae* and *C. brodyi.* (Note: only 6 individual bivalves were used for *Pfiesteria shumwayae* and *Cryptoperidiniopsis brodyi* experiments). The bivalves were also exposed to several thousand *G. catenatum* sexual resting cysts.

Following 48 h of exposure to the harmful algae, individual shellfish were thoroughly rinsed and transferred into 800 ml containers containing filtered seawater (35‰ salinity) to allow the shellfish to depurate and produce faeces. After 24 h, each bivalve was transferred to a new 800 ml containing filtered seawater for an additional 24 h allowing faecal material to be collected after 24 and 48 hours. The faecal pellets were examined under a light microscope (Zeiss Axioscop 2) for the presence of intact cells. Following this, approximately 0.25 ml of faecal suspension collected from each shellfish was inoculated into individual wells of 12-well flat bottom microplates containing 5 ml fresh nutrient medium to examine the ability of the microalgal cells to recover and subsequently grow. The growth of the microalgal cells was monitored

weekly using an inverted microscope (Zeiss Axiovert 25) for a period of four weeks. During this observation period, *Pfiesteria shumwayae* and *C. brodyi* were kept in the dark and were fed 1 ml of prey (*Rhodomonas* sp.) every 5 days.

7.4 Results

Intact microalgal cells of all species except *Chattonella marina* were found in the faecal pellets of the Pacific oysters after 24 h depuration in filtered seawater (Table 2). Oyster faeces produced after 48 h depuration in filtered seawater generally contained fewer intact algal cells. No *K. papilionacea* or *C. marina* cells were observed to be intact after 48 h depuration in filtered seawater (Table 2). Figure 1 shows intact *K. foliaceum* cells in oyster faecal material after 24 and 48 h depuration in filtered seawater.

No intact cells of *H. akashiwo* or *C. marina* were present in the faeces from the blue mussel after 24 and 48 h depuration in filtered seawater and intact *K. papilionacea* cells were only present in the faecal material after 24 h depuration (Table 2). Figure 2A shows faecal material from a *K. papilionacea*-fed mussel after 48 hours in filtered seawater. The material is thoroughly processed and no intact *K. papilionacea* cells are visible. Intact cells of the remaining species were present in the mussel faeces after 24 and 48 hours of depuration (Table 2).

Table 2. Presence of intact microalgal cells in the faecal material of Pacific oysters and blue mussels after 24 and 48 h of depuration in filtered seawater. (+: intact cells observed; -: intact cells not observed)

Microalgal species	Bivalve species			
	Crassostrea gigas		*Mytilus galloprovincialis*	
	24 h	48 h	24 h	48 h
Alexandrium catenella	+	+	+	+
Chattonella marina	-	-	-	-
Cryptoperidiniopsis brodyi	+	+	+	+
Gymnodinium catenatum	+	+	+	+
Heterosigma akashiwo	+	+	-	-
Karenia papilionacea	+	-	+	-
Kryptoperidinium foliaceum	+	+	+	+
Pfiesteria shumwayae	+	+	+	+

The density of microalgal cells present in the bivalve faecal pellets varied not only between the microalgae species, but also between individual shellfish. The majority of microalgal cells in the faecal material were non-motile, yet regained their motility once inoculated in GSe media. However, motile cells of *G. catenatum* and *K. papilionacea, P. shumwayae* and *C. brodyi* were observed (Fig. 2B). *Gymnodinium catenatum, A. catenella* and *K. foliaceum* were observed in the faecal pellets of the two bivalve species in two different morphologies. *Gymnodinium catenatum* was found both as vegetative cells and sexual resting cysts. The resting cyst walls appeared slightly bleached yet were still found to be viable (Fig. 3A). In the Pacific oyster trials, 17 *G. catenatum* resting cysts were recovered from faecal material after 48 h depuration. Of these, 11 cysts germinated into healthy swimming planozygote cells, which was less but comparable to the control (20 of 28). A similar result was found in the blue mussel trials, with successful germination achieved in 15 of the recovered 24 *G. catenatum* cysts. Temporary cysts dominated the cell type found in the *A. catenella* and *K. foliaceum* faecal material, yet vegetative cells were occasionally observed (Fig. 3B).

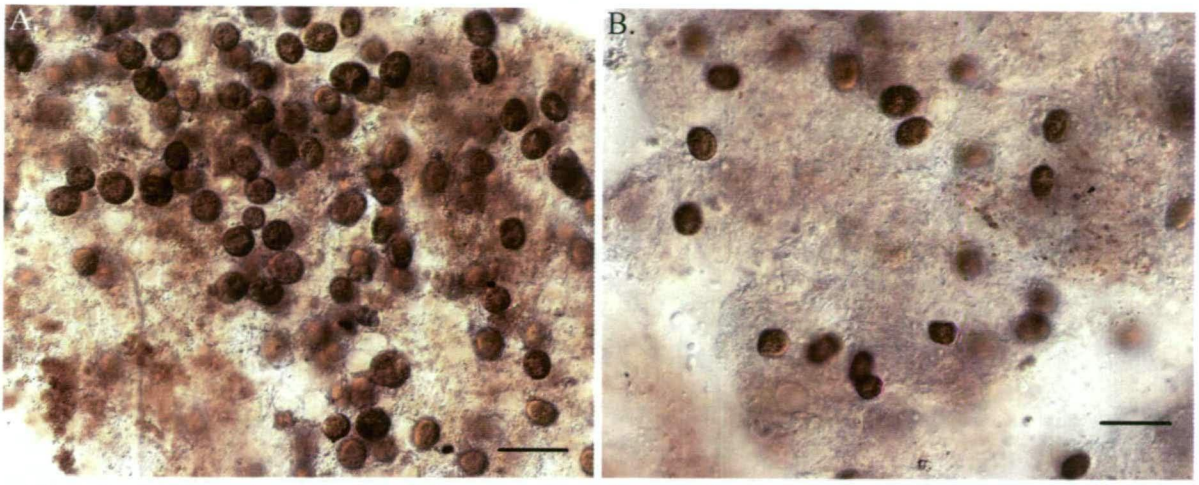

Fig. 1. Light micrograph of intact cells of *K. foliaceum* in Pacific oyster faecal material after (A) 24 and (B) 48 hours depuration in filtered seawater. Scale bars=50μm

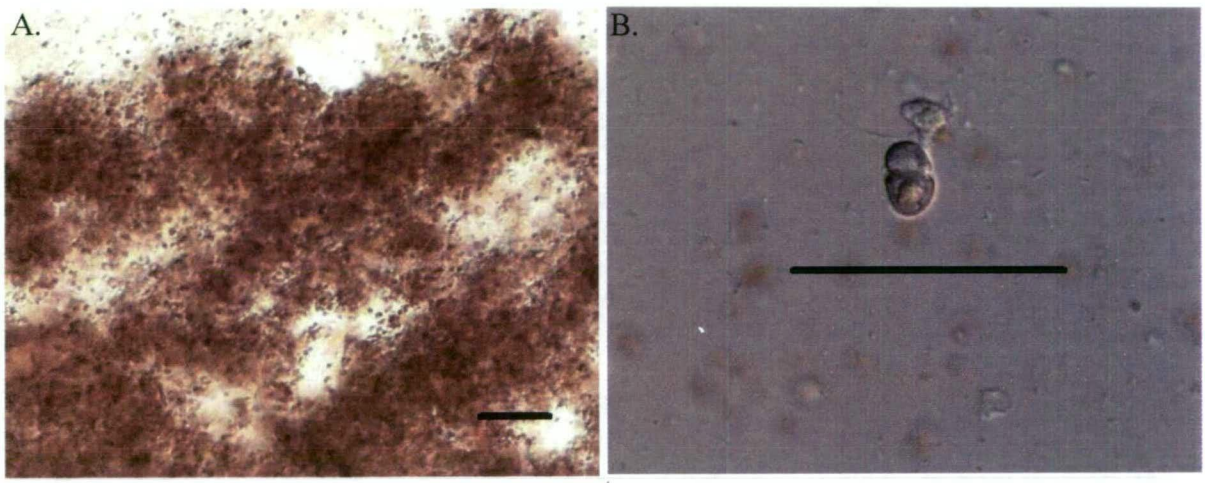

Fig. 2. Light micrograph showing (A) thoroughly processed faecal material from a *K. papilionacea*-fed mussel after 48 hours depuration in filtered seawater, and (B) a motile cell of *Pfiesteria shumwayae* in mussel faecal material after 24 hours depuration in filtered seawater. Scale bars=50μm

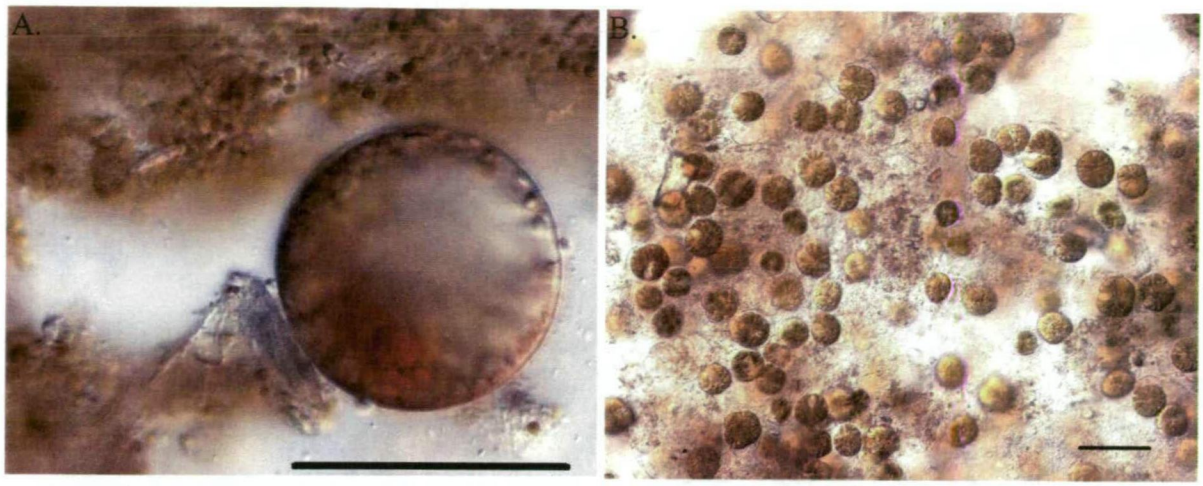

Fig.3. Light micrograph of (A) an intact *Gymnodinium catenatum* resting cyst in mussel faecal material, and (B) intact cells of *A. catenella* (temporary cysts) in oyster faeces after 48 hours depuration in filtered seawater. Scale bars=50μm

When the faecal suspensions were inoculated in GSe nutrient medium, growing populations were established for all the microalgal species that were observed as intact cells (Table 3). Some species were more successful at establishing growing populations than others. *Kryptoperidinium foliaceum, P. shumwayae* and *C. brodyi* were the most successful at establishing growing populations with cells able to recover in all the biodeposit trials for both the Pacific oyster and blue mussel, with the exception of the blue mussel trials that contained dead shellfish (Tables 3-6). On the other hand, only 30% of the *H. akashiwo* oyster trials resulted in growing populations (Tables 3 and 5). The ability of *G. catenatum* cells to recover into growing populations was less in the 48 h trials compared to the 24 h trials. Following 24 h depuration, 90% of the *G. catenatum* trials of both oysters and mussels recovered into growing populations (Table 3 and 4). This declined to 40% for the Pacific oyster (Table 5), and 20% for the blue mussel (Table 6) following 48 h depuration in filtered seawater.

The time taken for cells to recover once discharged in the bivalve faeces varied between the microalgal species. The culture plates inoculated with faecal pellets from bivalves exposed to *K. foliaceum* showed a recovery of cells within 1-3 days. Cells of *P. shumwayae, C. brodyi, K. papilionacea, H. akashiwo* and *G. catenatum* generally recovered within 1 week, whereas *A. catenella* cells took from 2 to 10 days to form detectable, growing populations.

Table 3. Regrowth of microalgal cells from the faecal material of the Pacific oyster (*Crassostrea gigas*) following 24 hours depuration in filtered seawater. Faecal material from each shellfish was inoculated into individual wells of 12-well flat bottom microplates containin GSe nutrient medium and was monitored for a period of 4 weeks.
(+ = regrowth; - = no regrowth)

Phytoplankton species	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Total (regrowth/total)
Alexandrium catenella	+	+	+	+	+	+	+	+	+	+	10/10
Chattonella marina	-	-	-	-	-	-	-	-	-	-	0/10
Cryptoperidiniopsis brodyi	+	+	+	+	+	+					6/6
Gymnodinium catenatum	-	+	+	+	+	+	+	+	+	+	9/10
Heterosigma akashiwo	-	-	-	+	+	-	-	-	+	-	3/10
Karenia papilionacea	-	+	+	+	+	-	-	+	+	-	6/10
Kryptoperidinium foliaceum	+	+	+	+	+	+	+	+	+	+	10/10
Pfiesteria shumwayae	+	+	+	+	+	+					6/6

Table 4. Regrowth of microalgal cells from the faecal material of the blue mussel (*Mytilus galloprovincialis*) following 24 hours depuration in filtered seawater. Faecal material from each shellfish was inoculated into individual wells of 12-well flat bottom microplates containin GSe nutrient medium and was monitored for a period of 4 weeks.
(+ = regrowth; - = no regrowth) (* denotes dead shellfish)

Phytoplankton species	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Total (regrowth/total)
Alexandrium catenella	+	+	-	+	-	+	+	-	-*	+	6/10
Chattonella marina	-	-	-	-	-	-	-	-	-	-	0/10
Cryptoperidiniopsis brodyi	+	+	+	+	+	+					6/6
Gymnodinium catenatum	+	+	+	+	+	+	+	+	-	+	9/10
Heterosigma akashiwo	-	-	-	-	-	-	-	-	-	-	0/10
Karenia papilionacea	-	-	+	+	-*	-	-	-	+	-	3/10
Kryptoperidinium foliaceum	+	+	+	+	+	+	+	+	+	+	10/10
Pfiesteria shumwayae	+	+	+	+	+	+					6/6

Table 5. Regrowth of microalgal cells from the faecal material of the Pacific oyster (*Crassostrea gigas*) following 48 hours depuration in filtered seawater. Faecal material from each shellfish was inoculated into individual wells of 12-well flat bottom microplates containin GSe nutrient medium and was monitored for a period of 4 weeks.
(+ = regrowth; - = no regrowth)

Phytoplankton species	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Total (regrowth/total)
Alexandrium catenella	+	+	+	+	+	+	+	+	+	-	9/10
Chattonella marina	-	-	-	-	-	-	-	-	-	-	0/10
Cryptoperidiniopsis brodyi	+	+	+	+	+	+					6/6
Gymnodinium catenatum	-	-	-	+	+	-	+	+	-	-	4/10
Heterosigma akashiwo	-	-	-	+	+	-	-	-	+	-	3/10
Karenia papilionacea	-	-	-	-	-	-	-	-	-	-	0/10
Kryptoperidinium foliaceum	+	+	+	+	+	+	+	+	+	+	10/10
Pfiesteria shumwayae	+	+	+	+	+	+					6/6

Table 6. Regrowth of microalgal cells from the faecal material of the blue mussel (*Mytilus galloprovincialis*) following 48 hours depuration in filtered seawater. Faecal material from each shellfish was inoculated into individual wells of 12-well flat bottom microplates containin GSe nutrient medium and was monitored for a period of 4 weeks.
(+ = regrowth; - = no regrowth) (* denotes dead shellfish)

Phytoplankton species	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Total (regrowth/total)
Alexandrium catenella	-	+	-	-	-*	+	+	-	-*	+	4/10
Chattonella marina	-	-	-	-	-	-*	-	-	-	-	0/10
Cryptoperidiniopsis brodyi	+	+	+	+	-*	+					5/6
Gymnodinium catenatum	+	-	-	-	-*	-*	-	+	-	-	2/10
Heterosigma akashiwo	-	-	-	-	-*	-*	-	-	-	-	0/10
Karenia papilionacea	-	-	-	-	-	-*	-*	-*	-	-	0/10
Kryptoperidinium foliaceum	+	+	+	+	+	+	-*	-*	+	+	8/10
Pfiesteria shumwayae	+	+	+	-*	+	+					5/6

7.5 Discussion

This study unambiguously demonstrates that viable microalgal cells can pass through the digestive tract of adult blue mussels and Pacific oysters. Dinoflagellate species were found to be more likely to pass through the gut intact, recover and establish growing populations, whereas the two raphidophyte species were the least successful. Out of the 6 dinoflagellate species tested, *Alexandrium catenella, Cryptoperidiniopsis brodyi, Kryptoperidinium foliaceum* and *Pfiesteria shumwayae* were the most successful species in terms of passing through the digestive tract intact and recovering into actively growing populations. The time taken for the microalgal cells to recover into growing populations once discharged in the bivalve faecal pellets varied from 1 to 10 days. *Kryptoperidinium foliaceum* was the fastest species to recover (1-3 days) whereas *A. catenella* cells took from 2 to 10 days to form detectable, growing populations. No intact *C. marina* cells were found in both the mussel or oyster translocation experiments, and viable *H. akashiwo* cells were not recovered from the blue mussel trials. Similar species-specific bivalve-microalgal responses have been reported by Hégaret *et al.* (2008). These authors fed several harmful algal species including the dinoflagellates *Alexandrium fundyense, Alexandrium monilatum* and the raphidophyte *Heterosigma akashiwo* to various species of bivalve molluscs (bay scallops *Argopectin irradians,* eastern oysters *Crassostrea virginica,* northern quahogs *Mercenaria mercenaria,* blue mussels *Mytilus edulis,* softshell clams *Mya arenaria,* green mussels *Perna viridis,* Manila clams *Venerupis phillipinarum*) and found that after 24 or 48 h depuration in filtered seawater, cells of the raphidophyte *H. akashiwo* were only present in the faecal material of bay scallops, northern quahogs and eastern oysters; whereas intact *Alexandrium* cells were present in the faecal material of the majority of species tested, with the exception of the softshell clam.

The species-specific responses identified in the present study may be due to differences between the microalgae, such as cell palatability, cell durability, cell size or toxin profiles of the algae. Shumway and Cucci (1987) propose that toxicity is an important factor in allowing *Alexandrium* cells to pass through the gut intact. Conversely, Laabir and Gentien (1999) found that toxicity is not important, and suggest that the cellulose theca plays a more crucial role. In the present study,

however, intact vegetative dinoflagellate cells of both armoured (*A. catenella, K. foliaceum*) and unarmoured (*C. brodyi, G. catenatum, K. papilionacea, P. shumwayae*) species were able to pass through digestive tract of adult bivalves.

Another possibility is that the species-specific bivalve-shellfish interactions could be due to responses by the shellfish themselves. Previous reports have shown shellfish to exhibit a variety of responses to the presence of harmful microalgae, ranging from normal filtration to decreased filtration and even complete avoidance (Bricelj *et al.*, 1993; Hégaret *et al.*, 2007).

Viable vegetative cells, temporary cysts and sexual resting cysts were found to survive gut passage in the two bivalve species tested. The temporary cysts produced by *A. catenella* are most likely to have formed during gut passage, as the shellfish were only fed vegetative cells. For *Kryptoperidinium foliaceum*, even though the shellfish were fed vegetative cells (~90%) and temporary cysts (~10%), the majority of vegetative cells must also have transformed into temporary cysts in the digestive tract, as such cysts dominated the cell type found in the faecal material (>95%). Many microalgae species overcome unfavourable environmental conditions by forming temporary cysts. Laabir *et al.* (2007) indicate that vegetative dinoflagellate cells are exposed to a range of chemical and mechanical constraints during gut transit in shellfish which promotes temporary cyst formation. These cysts are known to be considerably more resistant to adverse conditions than the vegetative cells and thus microalgal cells embedded in bivalve faecal material as temporary cysts could potentially lead to the contamination of pristine areas.

Sexual resting cysts are known to be extremely robust and have been documented to pass intact through the gut of other organisms including copepods and polychaete worms (Montresor *et al.*, 2003; Kremp *et al.*, 2003). In the present study, the viability of *Gymnodinium catenatum* cysts was not significantly reduced following passage through the digestive tract of Pacific oysters and blue mussels. Kremp *et al.* (2003) even indicate that enclosure in a faecal pellet may provide favourable conditions for cyst germination. As the survival time of resting cysts is much longer than that of temporary cysts (Garcés *et al.*, 2002; Lewis *et al.*, 1999), the risk of introducing microalgae into pristine areas would be the greatest when translocating

shellfish that have ingested resting cysts as the cysts can be excreted in the faeces and lay dormant in the sediment for many years until conditions become favourable for germination.

Several authors suggest that holding shellfish in filtered or sterilised seawater for various lengths of time prior to their translocation may be an effective way of purging the shellfish of toxic or nuisance microalgae. Scarratt *et al.* (1993) retained mussel and scallop spat in a UV-irradiated, recirculating seawater system for a period for 12 hours and indicate that this would likely be a sufficient purging regime. Similarly, Hégaret *et al.* (2008) suggest that keeping adult bivalves in filtered seawater for a period of 24 to 48 hours before relocation allows the shellfish to clear its gut of viable algal cells. Yet, in the present experiment, intact cells of the majority of microalgal species tested were found in the faecal material of *C. gigas* and *M. galloprovincialis* after 48 hours depuration in filtered seawater, and indeed many of these cells recovered into growing populations. As the shellfish species used by Scarratt *et al.* (1993) and Hégaret *et al.* (2008) were exposed to similar algal bloom concentrations to those used in the present study, the duration of the purging and depuration strategies suggested by these authors are not long enough for Pacific oysters and blue mussels to completely clear their gut contents of all viable microalgal species. Further work is required to determine an effective mitigation strategy to eliminate the risk of introducing harmful microalgae via the translocation of the blue mussel and Pacific oyster. Potential treatment options may include freshwater immersion, longer periods of depuration in filtered seawater, heat or chemical treatment.

In summary, results generated from the present experiment demonstrate that bivalve shellfish are a probable pathway for transporting harmful microalgal species into new areas. As the shellfish-microalgal responses observed were species-specific, a methodical approach is required to test for certain shellfish and microalgal species that may present a risk. Microalgal species of greatest concern include toxic species that are capable of forming temporary or resting cysts such as the dinoflagellates *Alexandrium catenella* and *Gymnodinium catenatum*, which are often responsible for paralytic shellfish poisoning (PSP) outbreaks throughout the world. Further investigations are required to 1) determine the time needed for the complete

elimination of viable microalgal cells in the digestive tract of the bivalve species; and 2) determine an efficient, cost effective and safe treatment method to mitigate the risk of harmful microalgal introductions through the relocation of bivalve shellfish.

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Chapter 8

Potential treatment options to mitigate the risk of harmful microalgal introductions from translocation of juvenile and adult Pacific oysters

8.1 Abstract

Pacific oysters (*Crassostrea gigas*) are commonly transferred from one water body to another for outgrowing purposes or to establish new fisheries. Shellfish translocations pose a risk of introducing harmful or nuisance microalgal species to new areas as viable cells are retained in the digestive tract of molluscs and are capable of establishing populations once discharged in faeces. Several treatment options are currently used or are being considered by shellfish farmers to eliminate associated predators and fouling species such as *Undaria pinnatifida, Asterias amurensis* and *Carcinus maenas* as well as pathogens that might be associated with translocated shellfish; however very little attention has been given to eliminating, or minimising, phytoplankton associated with live oyster transfers. This study assessed whether immersion in freshwater, depuration in seawater or chemical biocide treatment can eliminate dinoflagellate cells following their ingestion by Pacific oysters. Depuration in filtered seawater for a period of 7 days was identified as the only treatment option capable of purging *Crassostrea gigas* adults and spat of viable dinoflagellate cells. No viable *Gymnodinium catenatum* cells were recovered from the oyster faeces after 4 days of depuration in filtered seawater and viable *Alexandrium catenella* and *Kryptoperidinium foliaceum* were not present following a depuration period of 7 days. This lengthy depuration period is likely to be incompatible with current oyster farming practices and may impact the condition of the shellfish. Dinoflagellate cells were not eliminated from the digestive tract of adult and spat Pacific oysters by immersion in freshwater for a period of 24 h. Intact cells of the three test species were observed in faecal material collected from adult and spat oysters after 24 h immersion in freshwater and 24 h depuration in filtered seawater. When exposed to hydrogen peroxide for 48 h, viable *G. catenatum* cells were not recovered from the 400 or 600 ppm spat treatments, however, the faecal material from several adult *C. gigas* exposed to 400 ppm contained viable cells. Viable cells were found in the faeces of all of the *A. catenella* and *K. foliaceum*-fed adult oyster and spat trials exposed to hydrogen peroxide, with the exception of the 400 ppm *A. catenella* trials (90%), after 48 h exposure. When the shellfish were exposed to chlorine dioxide at concentrations of 40 and 60 ppm for 48 h, all the *K. foliaceum*-fed oysters produced faecal material containing viable cells. Intact *A. catenella* cells that were capable of regrowth were recovered from 60 and 80% of the

40 and 60 ppm trials, respectively, and all the spat trials were found to produce faecal material containing viable cells. Viable *G. catenatum* cells were excreted in 40% of the chlorine dioxide-treated adult oysters; whereas the faecal material collected from the spat trials contained no viable cells. Although hydrogen peroxide and chlorine dioxide failed to eliminate dinoflagellate cells in the digestive tract of the Pacific oyster, both biocides may provide effective treatment options for sterilising faecal waste from a shellfish depuration plant. Further research should investigate alternative or more convenient treatment options to minimise the risk of toxic microalgal introductions through the relocation of Pacific oysters. Until an effective treatment option is discovered, the geographic translocation of Pacific oyster stocks should be regulated to minimise risks.

Keywords: Aquaculture, Harmful microalgae, Introductions and transfers, Pest management, *Crassostrea gigas*

8.2 Introduction

Oysters have been transported around the world to establish new fisheries for hundreds of years (Chew, 1990). The first documented case of large-scale oyster transfers dates back to as early as 1714 in Europe, when seed oysters were imported from Denmark for introduction in the western Dutch Wadden Sea (Wolff and Reise, 2002). Since then, large quantities of various oyster species have been transported to many parts of the world and have become the major organism used in shellfish aquaculture operations. Furthermore, their geographical range will no doubt continue to increase as oyster species are introduced into new regions.

Commercial oyster translocations can be an important vector for the introduction of non-indigenous organisms. In Europe, the translocation of oysters is considered to be a dominant source of non-indigenous species introductions, historically contributing to the introduction of at least as many species as international shipping operations (McKindsey *et al.,* 2007). Apart from the possibility of the translocated oysters themselves becoming invasive, live oysters consignments are known to harbour a wide variety of other organisms, which if introduced into sensitive areas used for aquaculture, can lead to negative effects on both the fishery itself and the marine environment. McKindsey *et al.* (2007) highlight three major classes of non-indigenous species of concern in relation to oyster transfers for aquaculture: 1) exotic macrospecies, such as seaweeds and zooplankton; 2) exotic phytoplankton (toxic and otherwise); and 3) exotic disease causing organisms. These organisms may occur on the shell surface; in the mantle cavity, digestive tract or the soft tissue of the oysters; or associated with sediment on the exterior surface or inside the shellfish (Minchin, 1996).

Several elimination, or preventative, treatment techniques are used to reduce the abundance of organisms transported with live shellfish transfers. These include immersion in freshwater, hot water and brine (Mineur *et al.,* 2007); periodical drying; and seawater depuration systems including the application of chlorine or UV irradiation (Richards, 1988). At present, shellfish farmers are also considering using chemical treatments, such as chlorine or hydrogen peroxide. Immersion in freshwater, hot water or brine, as well as chemical treatment, focuses solely on

reducing the number of organisms that occur on the shell surface of the oysters, such as seaweeds, zooplankton, bivalves and other fouling organisms. Seawater depuration systems, on the other hand, have been used since the late 1880's to reduce the levels of bioconcentrated bacteria and viruses within the shellfish (Richards, 1988). Very little attention has been given to eliminating, or minimising, phytoplankton associated with live shellfish transfers.

It is now widely acknowledged that translocated oysters are potential carriers of phytoplankton between aquaculture sites. Several recent studies have determined that viable microalgal cells (including toxic species) have the ability to survive gut passage in oysters and can establish viable populations once discharged in faecal material (Carricker, 1992; Laabir and Gentien, 1999; Laabir *et al.,* 2007; Hégaret *et al.,* 2008). Additionally, dinoflagellate cysts and other resting stages may also be present in the sediment and detritus contained inside shells (Minchin, 1996). The introduction of harmful microalgal species into sensitive areas used for aquaculture can result in vast ecological problems, economic loss, and even human health problems due to exposure to toxins from microalgae through consumption of contaminated shellfish (Hallegraeff, 1992; Burkholder, 1998). Given this, it is surprising that so little attention has been given to investigating potential treatment options or preventative techniques to mitigate the risk of harmful microalgal introductions through the movement of oysters. The extent to which the current treatment techniques used by shellfish farmers may reduce this risk is unknown.

Here, we examine the effectiveness of several potential treatment options for eliminating ingested marine microalgae in the Pacific oyster. *Crassostrea gigas* was chosen as the test organism as it is one of the most commercially important bivalve shellfish species worldwide due to its ability to thrive under a wide range of environmental conditions. It is estimated that 98% of the worlds total cultured oyster production involves *C. gigas* (Love and Langenkamp, 2003) and several authors believe that the introduction of *C. gigas* into new regions for aquaculture is one of the greatest vectors for the introduction of non-indigenous marine organisms worldwide (Wolff and Reise, 2002; McKindsey *et al.,* 2007). In one study, for example, a live consignment of Pacific oysters exported from France to Ireland contained 67 species of microalgae (43 diatoms, 22 dinoflagellates and 2

silicoflagellates) including 15 types of dinoflagellate cysts, even though the oysters were deemed to be free of other organisms (O'Mahoney, 1993).

In Australia, Pacific oyster farming first began in the 1960's in Tasmania (Nell, 2003), although the species was originally introduced into Pittwater in the late 1940's (Medcof and Wolf, 1975). Current intensive farming methods involve the continuous transfer of oyster spat from nursery areas to the aquaculture sites due to the natural recruitment of *C. gigas* larvae being unreliable and inconsistent. This movement of oyster spat occurs on both a regional and national scale, however no formal regulatory measures exist to minimise the risk of accidental microalgal introductions through shellfish translocations. In addition, live Australian Pacific oysters are also exported to Asia, Europe and North America for seafood, further increasing the potential for accidental microalgal introductions. The aim of the present experiment, therefore, was to determine whether depuration in filtered seawater, freshwater immersion or chemical biocide treatment (hydrogen peroxide, chlorine dioxide) can eliminate the dinoflagellates *Alexandrium catenella, Gymnodinium catenatum* and *Kryptoperidinium foliaceum* following their ingestion by *C. gigas* adults and spat.

8.3 Materials and methods

Biological materials and culture methods

Adult Pacific oysters (*C. gigas*) ranging in size from 69-85 mm shell length were obtained from Barilla Bay Oysters (Orielton Lagoon, SE Tasmania). Pacific oyster spat ranging in size from 11-45 mm shell length were supplied by Oyster Bay Oysters (Little Swanport, Tasmania). Adult oysters and spat were fed three cultured dinoflagellate species obtained from the microalgal culture collection at the School of Plant Science, University of Tasmania.

Vegetative cultures of the dinoflagellates *Gymnodinium catenatum* (strain GCTRA01), *Alexandrium catenella* (strain ACSHO2) and *Kryptoperidinium foliaceum* (strain KFCRO1) were grown in 5-20 litre culture flasks containing GSe nutrient medium (28‰ salinity) (Blackburn *et al.*, 1989). Nutrient medium was made with filtered seawater (35‰ salinity) collected from Bruny Island, Tasmania.

Seawater was filtered using a 0.2μm membrane filter. All cultures were grown at 17°C under 12h dark/ 12h light. Light was provided at an intensity of 100 μmol quanta $m^{-2}s^{-1}$ by a bank of cool-white fluorescent tubes.

Feeding experiment

Two-hundred and forty Pacific oyster adults and approximately 650 spat individuals were placed in a 200 L tank containing filtered seawater (35‰ salinity) for 48 h at 14°C to allow them to depurate. Aeration was provided by two 4-way aquarium bubblers. Following depuration, 80 adult oysters and over 200 spat individuals were transferred to three 80 L tanks and were exposed separately to vegetative cells of the three dinoflagellate species (*Gymnodinium catenatum*, *Alexandrium catenella*, *Kryptoperidinium foliaceum*) for 48 h at concentrations equivalent to a natural bloom (10^6 cells L^{-1}) (Kempton *et al.*, 2002; Collos *et al.*, 2004; Rodriguez *et al.*, 2005) (Note: *K. foliaceum* cultures also contained temporary cysts). Following 24 h, no dinoflagellate cells were visible in the water column; therefore the shellfish were fed again. After 48 h of exposure to the simulated bloom, shellfish were removed, rinsed thoroughly in filtered seawater, and transferred to the treatment experiments. All treatment experiments were conducted at 14°C.

Seawater depuration experiments

Individual Pacific oyster adults (10 trials) and spat (5 trials each containing 4 individuals) from each of the three dinoflagellate feeding experiments were transferred into 800 ml plastic containers containing filtered seawater (35‰ salinity) to allow the shellfish to depurate and produce faeces. Every 24 h, faecal material from each trial was collected and the oysters were rinsed, and transferred to new 800 ml containers containing filtered seawater. This procedure was repeated for 7 days. At each 24 h interval, the faecal material was examined under a light microscope (Zeiss Axioscop 2) for the presence of intact cells and approximately 0.25 ml of faecal suspension collected from each trial was inoculated into 12-well culture plates containing 5 ml of GSe nutrient medium to examine the ability of the microalgal cells to recover and subsequently grow. The regrowth of the harmful algal cells was

monitored daily for a period of 2 weeks using an inverted microscope (Zeiss Axiovert 25).

Freshwater immersion experiments

Ten adult oysters and 20 spat from each of the three dinoflagellate feeding experiments were transferred to separate 10 L tanks containing 8 L of freshwater (milli-Q) for a period of 24 h. Following the 24 h freshwater treatment period, the shellfish were rinsed and individual Pacific oyster adults (10 trials) and spat (5 trials each containing 4 individuals) were transferred to 800 ml plastic containers containing filtered seawater (35‰ salinity) to allow the shellfish to depurate and produce faeces. Faecal material was recovered, examined for intact cells and inoculated into GSe nutrient medium to assess regrowth every 24 h for a period of 6 days using the methods described for the seawater depuration experiment.

Chemical biocides

The stock solution of Hydrogen peroxide was a newly opened bottle of 30% w/v hydrogen peroxide solution (Pronalys AR*, Biolab (Aust) Limited). The chlorine dioxide biocide, BioSAFE 5 (BioSAFE Technologies, Australia), was supplied at a concentration of 30,000 ppm sodium chlorite. BioSAFE 5 uses a process known as acidification of sodium chlorite to form chlorine dioxide. In the present study, BioSAFE 5 was applied using a 5:1 ratio of BioSAFE 5 to citric acid. This activation method converts approximately 20% of the sodium chlorite to chlorine dioxide and 70% to chlorous acid. All BioSAFE 5 concentrations listed in the present experiment are expressed as the level of free-chlorine. Free-chlorine measurements were taken using a DPD 1 chlorine test kit (Magnor®).

Chemical treatment of vegetative dinoflagellate cells and temporary cysts

Prior to exposing the shellfish to the chemical biocides, the effectiveness of both biocides was tested against vegetative cells of the three test microalgal species and temporary cysts of *K. foliaceum.* For the treatment of vegetative cells, one millilitre

of each vegetative dinoflagellate culture (*A. catenella, G. catenatum, K. foliaceum*) was placed into 24-well flat bottom microplates and various concentrations of each biocide were applied. Microalgal cultures used were 2 weeks old and contained approximately 1-2 $x10^6$ cells per litre for *A. catenella* and *G. catenatum*; and 4-$5x10^6$ cells per litre for *K. foliaceum*. Hydrogen peroxide was applied at concentrations of 5, 10, 25, 50, 100 and 200 ppm. For BioSAFE 5, concentrations of 0.1, 0.25, 0.5, 1, 2, 3, 4, 5, 10 and 20 ppm were applied. All biocide concentrations were prepared in filtered seawater (35‰ salinity). As per the manufacturers' instructions, BioSAFE 5 was combined with the citric acid activator and left for 10 min prior to its addition to the seawater. Each biocide and control treatment was replicated three times. Culture plates were sealed with parafilm following the biocide application and placed under a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ at 17°C. Cell viability was assessed after 24 h by comparing the percentage of non-viable cells between the various biocide concentrations and a control treatment. Motility, flagellar movement, and cell wall and organelle integrity were used as indicators of viability. Viability observations were conducted on a Zeiss inverted microscope (Zeiss Axiovert 25).

For the treatment of *K. foliaceum* temporary cysts, cysts were placed in 12-well flat bottom microplates containing 2 ml of filtered seawater and various concentrations of the two biocides were subsequently applied. Each trial was replicated three times. After the application of the biocide, the culture plates were sealed with parafilm and placed under culture conditions at 17°C. After 24 h exposure, the temporary cysts were removed, washed in sterile GSe medium, and transferred to new 12 well culture plates containing fresh GSe media. The treated cysts were then placed back under culture conditions with a light intensity of 100 µmol quanta $m^{-2}s^{-1}$ at 17°C with a 12h dark/ 12h light photoperiod. Treatments were examined daily for cyst germination. Germination observations were conducted on a Zeiss inverted microscope (Zeiss Axiovert 25).

Chemical treatment of shellfish

Five adult oysters and 20 spat individuals from each of the dinoflagellate feeding experiments were placed in separate 10 L tanks containing 8 L of filtered seawater (35‰ salinity). This procedure was replicated eight times allowing the shellfish to be

treated at two different concentrations of each biocide for exposure periods of 24 and 48 h. Shellfish were exposed to hydrogen peroxide at concentrations of 200 and 400 ppm for 24 h; and 400 and 600 ppm for 48 h. The chlorine dioxide biocide, BioSAFE 5, was applied at concentrations of 20 and 40 ppm for 24 h; and 40 and 60 ppm for 48 h.

In an attempt to determine the concentrations of the biocides that would be required to kill Pacific oysters, five adults and 10 spat individuals from the *K. foliaceum* feeding experiment were exposed to extremely high biocide concentrations for a period of 96 h. Hydrogen peroxide was applied at 2000 and 4000 ppm; and BioSAFE 5 was applied at concentrations of 200 and 400 ppm.

Following treatment with the chemical biocides, the bivalves were removed, rinsed in filtered seawater, and individual adults (5 trials) and groups of 4 spat individuals (5 trials) from each treatment were transferred to 800 ml containers containing filtered seawater (35‰ salinity) to allow the shellfish to depurate and produce faeces. The presence of intact cells and ability of the cells to recover into growing populations was assessed following 24 h depuration in filtered seawater using the methods described in the seawater depuration experiments.

8.4 Results

Seawater depuration experiments

Gymnodinium catenatum cells were eliminated from the digestive tract of adult and spat Pacific oysters following a depuration period of 4 days in filtered seawater. Figure 1 shows faecal material collected from *G. catenatum*-fed Pacific oysters after 24, 48 and 96 h of depuration in filtered seawater. After 24 and 48 h of depuration, faecal material collected from the adult oysters contained intact *G. catenatum* cells in every trial; and 90% of the trials resulted in viable cultures when the faecal material was inoculated in GSe nutrient medium (Table 1). Intact *G. catenatum* cells and subsequent regrowth occurred in 50% of the adult trials after 72 h. Only 1 of the 10 adult oysters was found to have intact cells in its faeces following a 96 h depuration period (Fig. 1E); and these cells failed to produce viable cultures (Table 1). Spat

oysters produced faeces containing intact *G. catenatum* cells that were capable of regrowth after 24, 48 and 72 h of depuration in filtered seawater (Table 2). No *G. catenatum* cells were observed in the spat trials after 3 days yet cell regrowth was detected in one trial (Table 2). Viable *G. catenatum* cells were not observed in spat faeces after 96 h of depuration (Fig. 1F).

Cells of *A. catenella* and *K. foliaceum* were capable of surviving in the digestive tract of the Pacific oysters for a considerably longer period than those of *G. catenatum.* Following 6 days of depuration in filtered seawater, 40% of the oyster trials still produced faeces that contained cells of *A. catenella* that could produce viable cultures, and the faecal material recovered from oysters that were fed *K. foliaceum* contained viable cells capable of regrowth in 60% of the spat trials and 50% of the adult shellfish (Tables 1 and 2). Seven days of depuration in filtered seawater was required before intact *A. catenella* and *K. foliaceum* cells were not observed (Fig. 2 and 3 E, F) and no regrowth occurred when the faecal material was inoculated in nutrient medium (Tables 1 and 2). Microscopic observations of the faeces showed that the material contained much higher densities of intact cells of A. *catenella* and *K. foliaceum* compared to the *G. catenatum* trials (Fig. 2 and 3). For example, the faecal material produced by *K. foliaceum*-fed oysters was composed almost entirely of algal cells following 24 h of depuration (Fig. 3A, B). The majority of *A. catenella* and *K. foliaceum* cells observed in the oyster faecal material were in the form of temporary cysts (Fig. 2 and 3).

Table 1. Summary of results from the seawater depuration experiments on adult Pacific oysters. The table shows total numbers of adult oysters that produced faecal material containing intact cells of the microalgal species (number of trials containing intact cells/total trials); and the total number of trials in which microalgal regrowth occurred from the shellfish faecal deposits (number of trials where regrowth of microalgal cells occurred/total trials). Faecal material was collected from each individual shellfish (10 trials) at 24 h intervals and examined microscopically for the presence of intact microalgal cells. Faecal material was inoculated into GSe medium to assess the ability of the microalgal cells to recover into growing populations.

Dinoflagellate species	24		48		72		96		120		144		168	
	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth
Alexandrium catenella	10/10	10/10	10/10	10/10	10/10	8/10	7/10	7/10	4/10	3/10	5/10	4/10	0/10	0/10
Gymnodinium catenatum	10/10	9/10	10/10	9/10	5/10	5/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Kryptoperidinium foliaceum	10/10	10/10	10/10	9/10	7/10	6/10	8/10	6/10	7/10	3/10	6/10	5/10	2/10	0/10

Table 2. Summary of results from the seawater depuration experiments on Pacific oyster spat. The table shows total numbers of spat trials that produced faecal material that containing cells of the microalgal species (number of trials containing intact cells/total trials); and the total number of trials in which microalgal regrowth occurred from the shellfish faecal deposits (number of trials where regrowth of microalgal cells occurred/total trials). Faecal material was collected from each spat trial (5 trials) at 24 h intervals and examined for the presence of intact microalgal cells. Faecal material was inoculated into GSe medium to assess the ability of the microalgal cells to recover into growing populations.

Dinoflagellate species	24		48		72		96		120		144		168	
	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth
Alexandrium catenella	5/5	4/5	5/5	5/5	5/5	5/5	4/5	4/5	4/5	3/5	2/5	2/5	0/5	0/5
Gymnodinium catenatum	4/5	2/5	4/5	3/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Kryptoperidinium foliaceum	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5	4/5	3/5	2/5	0/5

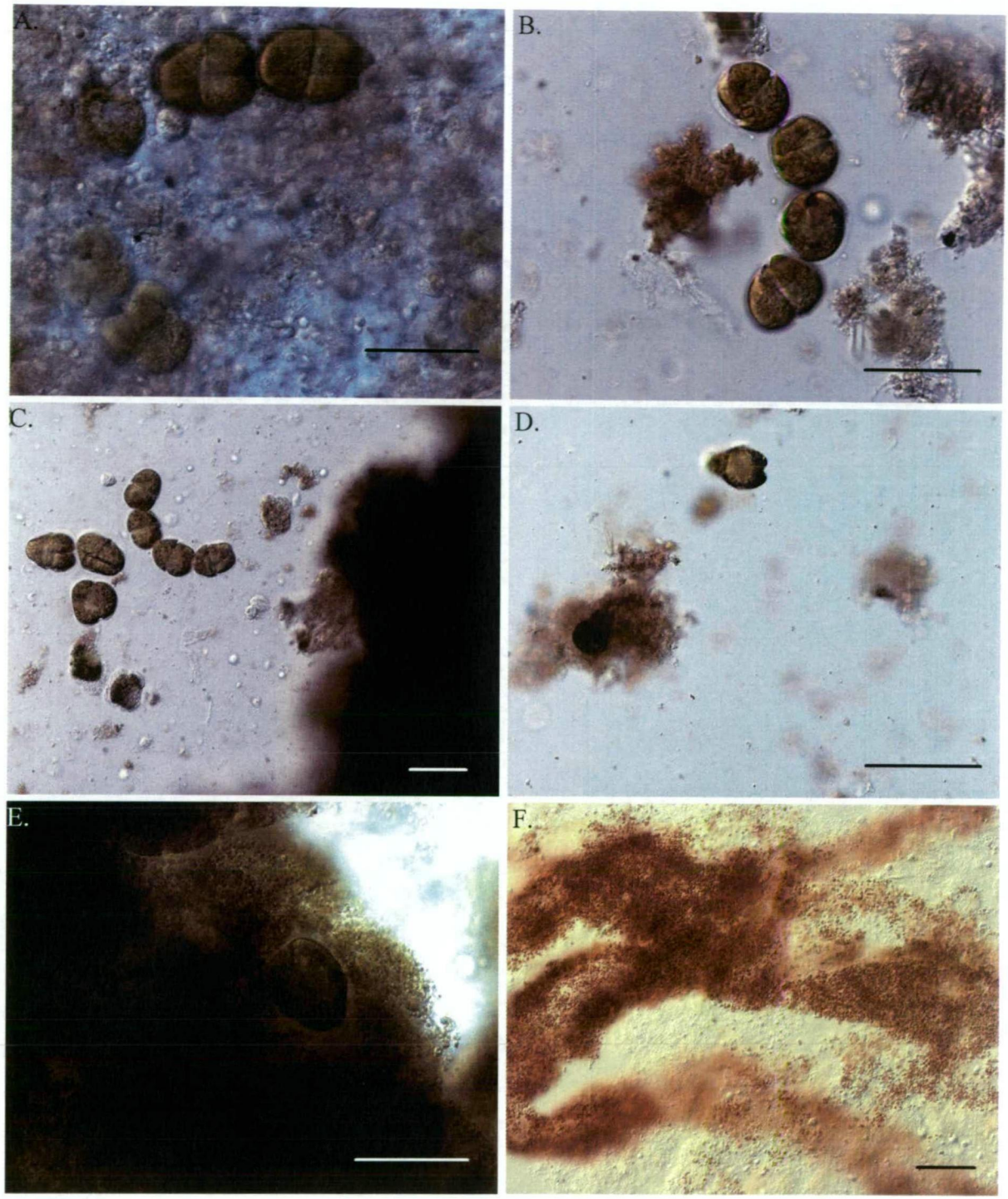

Fig. 1. Light micrographs of faecal material produced by Pacific oysters after being exposed to *Gymnodinium catenatum* and then transferred to filtered seawater for depuration (seawater depuration experiment). (A) Adult oyster (24 h depuration): (B) Spat oyster (24 h depuration): (C) Adult oyster (48 h deputation): (D) Spat oyster (48 h depuration): (E) Adult oyster (96 h depuration): (F) Spat oyster (96 h depuration). Scale bars=50 μm

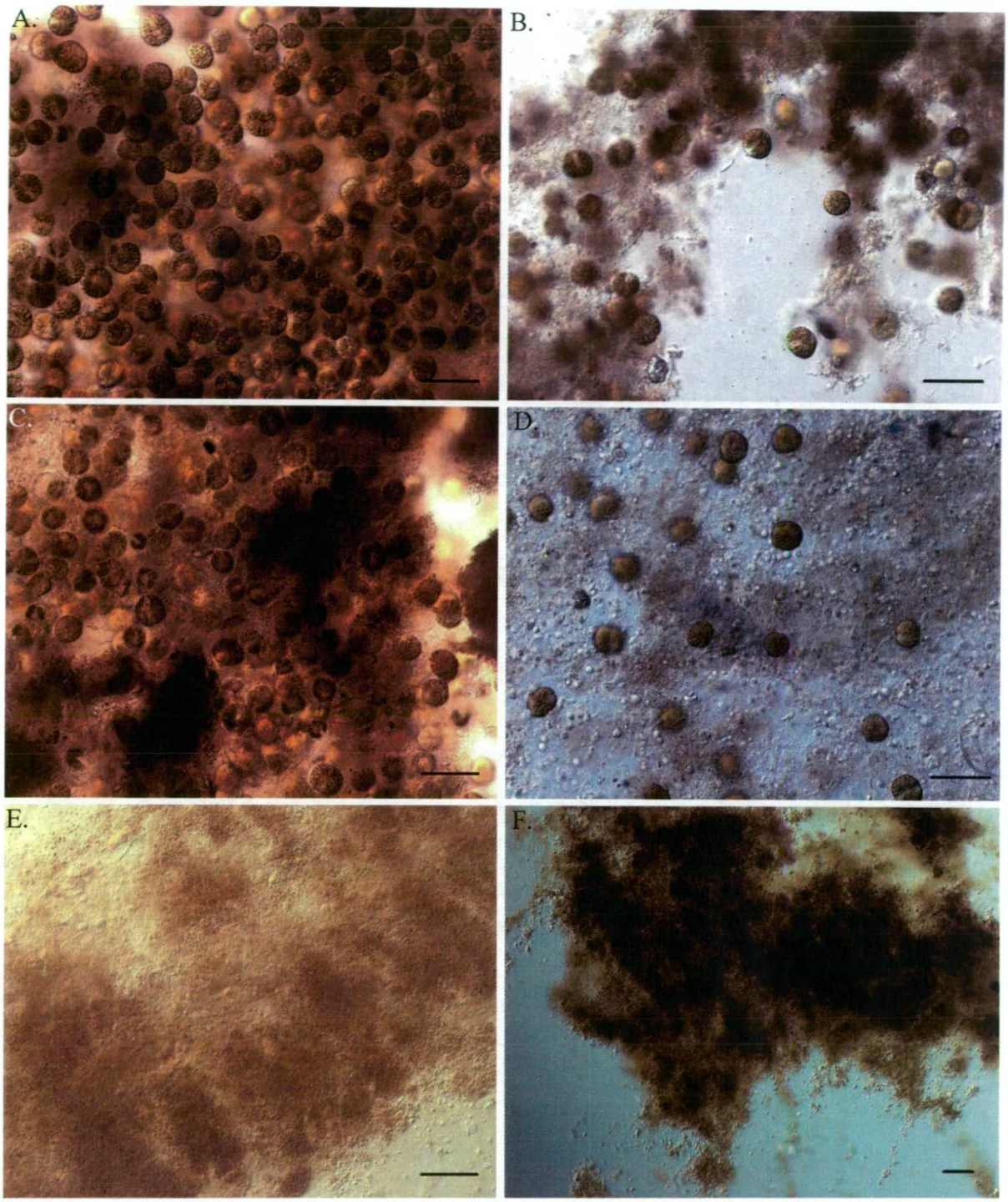

Fig. 2. Light micrographs of faecal material produced by Pacific oysters after being exposed to *Alexandrium catenella* and then transferred to filtered seawater for depuration (seawater depuration experiment). (A) Adult oyster (24 h depuration): (B) Spat oyster (24 h depuration): (C) Adult oyster (96 h deputation): (D) Spat oyster (96 h depuration): (E) Adult oyster (144 h depuration): (F) Spat oyster (144 h depuration). Scale bars=50 μm

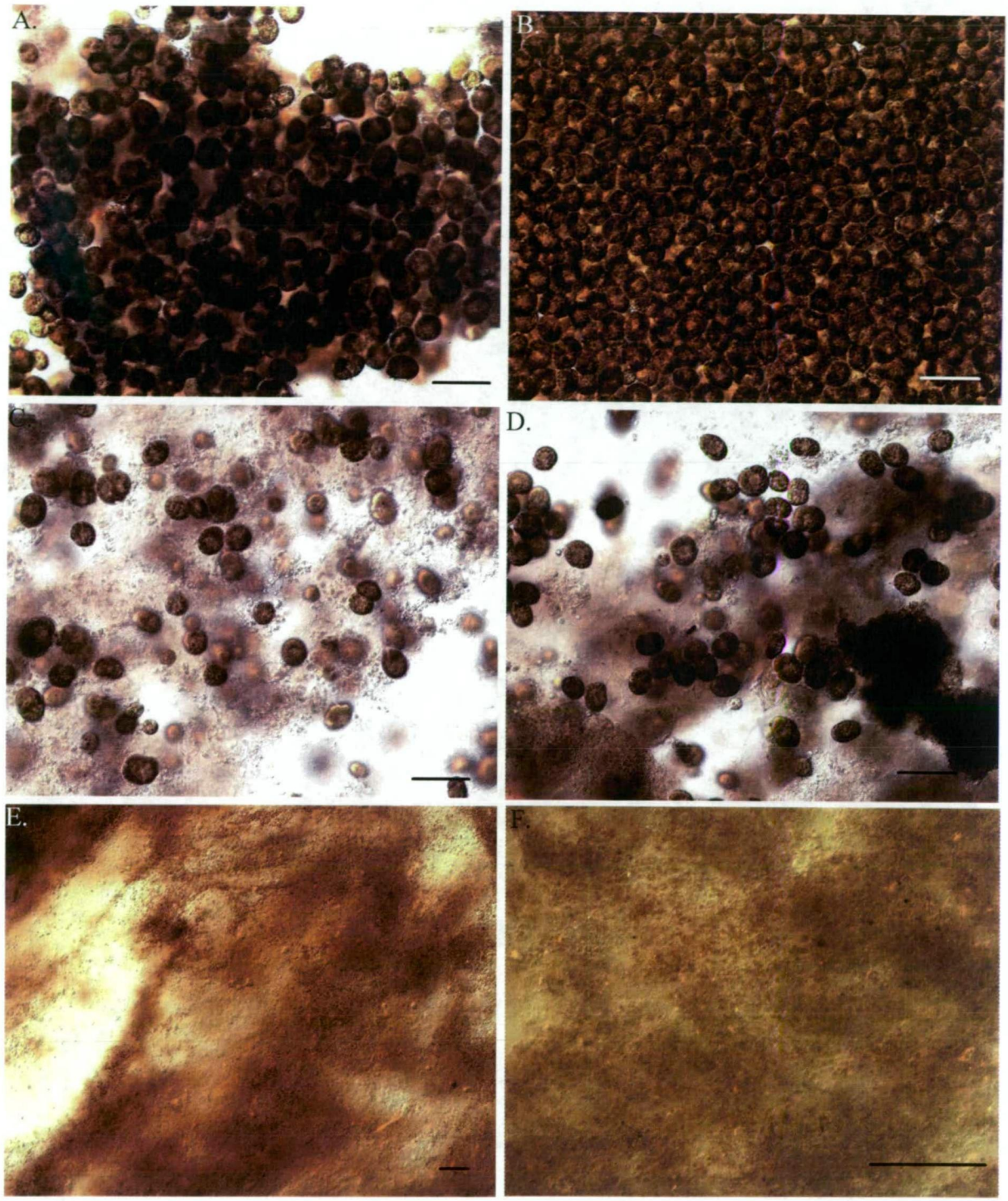

Fig. 3. Light micrographs of faecal material produced by Pacific oysters after being exposed to *Kryptoperidinium foliaceum* and then transferred to filtered seawater for depuration (seawater depuration experiment). (A) Adult oyster (24 h depuration): (B) Spat oyster (24 h depuration): (C) Adult oyster (96 h deputation): (D) Spat oyster (96 h depuration): (E) Adult oyster (144 h depuration): (F) Spat oyster (144 h depuration). Scale bars=50 μm

Freshwater immersion experiments

The results of the freshwater immersion experiments are presented in Table 3 for the adult oyster trials and Table 4 for the spat trials. Immersing oysters in freshwater for a period of 24 h failed to eliminate viable algal cells from the digestive tract of the shellfish. Intact cells of all three dinoflagellate species were observed in faecal material collected from adult and spat oysters after 24 h immersion in freshwater and 24 h depuration in filtered seawater (Fig. 4). Intact cells of *A. catenella* and *K. foliaceum* were present in every adult and spat trial, and the majority of these cells produced viable cultures when inoculated in nutrient medium (Tables 3 and 4). Following 24 h immersion in freshwater and 24 h depuration in filtered seawater, *G. catenatum* cells isolated from the bivalve faecal material recovered in 70% of the adult trials, and 60% of the spat trials. Intact *G. catenatum* cells were present in the faeces following 24 h immersion in freshwater and 72 h depuration in seawater but these cells failed to establish viable cultures (Tables 3 and 4). No regrowth of *Alexandrium catenella* cells from the faecal material produced by the adult shellfish was observed following an additional 6 days of seawater depuration (Table 3), however, the faecal material from one of the spat trials still contained viable cells (Table 4). Regrowth of *K. foliaceum* cells occurred in 30 % of the adult shellfish and 40% of the spat trials following a period of 24 h immersion in freshwater and 6 days depuration in filtered seawater.

Table 3. Summary of results from the freshwater immersion experiments on adult Pacific oysters. The table shows total numbers of adult oysters that produced faecal material containing intact cells of the microalgal species (number of trials containing intact cells/total trials); and the total number of trials in which microalgal regrowth occurred from the shellfish faecal deposits (number of trials where regrowth of microalgal cells occurred/total trials). Faecal material was collected from each individual shellfish (10 trials) at 24 h intervals following the initial 24 h freshwater treatment period and examined microscopically for the presence of intact microalgal cells. Faecal material was inoculated into GSe medium to assess the ability of the microalgal cells to recover into growing populations.

Dinoflagellate species	24		48		72		96		120		144	
	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth
Alexandrium catenella	10/10	10/10	10/10	7/10	9/10	7/10	5/10	2/10	3/10	3/10	1/10	0/10
Gymnodinium catenatum	6/10	7/10	6/10	4/10	3/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Kryptoperidinium foliaceum	10/10	10/10	10/10	10/10	10/10	10/10	10/10	6/10	6/10	7/10	3/10	3/10

Table 4. Summary of results from the freshwater immersion experiments on Pacific oyster spat. The table shows total numbers of spat trials that produced faecal material containing intact cells of the microalgal species (number of trials containing intact cells/total trials); and the total number of trials in which microalgal regrowth occurred from the shellfish faecal deposits (number of trials where regrowth of microalgal cells occurred/total trials). Faecal material was collected from each spat trial (5 trials) at 24 h intervals following the initial 24 h freshwater treatment period and examined for the presence of intact microalgal cells. Faecal material was inoculated into GSe medium to assess the ability of the microalgal cells to recover into growing populations.

Dinoflagellate species	24		48		72		96		120		144	
	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth
Alexandrium catenella	5/5	5/5	5/5	5/5	5/5	5/5	4/5	3/5	3/5	1/5	1/5	1/5
Gymnodinium catenatum	5/5	3/5	5/5	2/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Kryptoperidinium foliaceum	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5	3/5	4/5	2/5

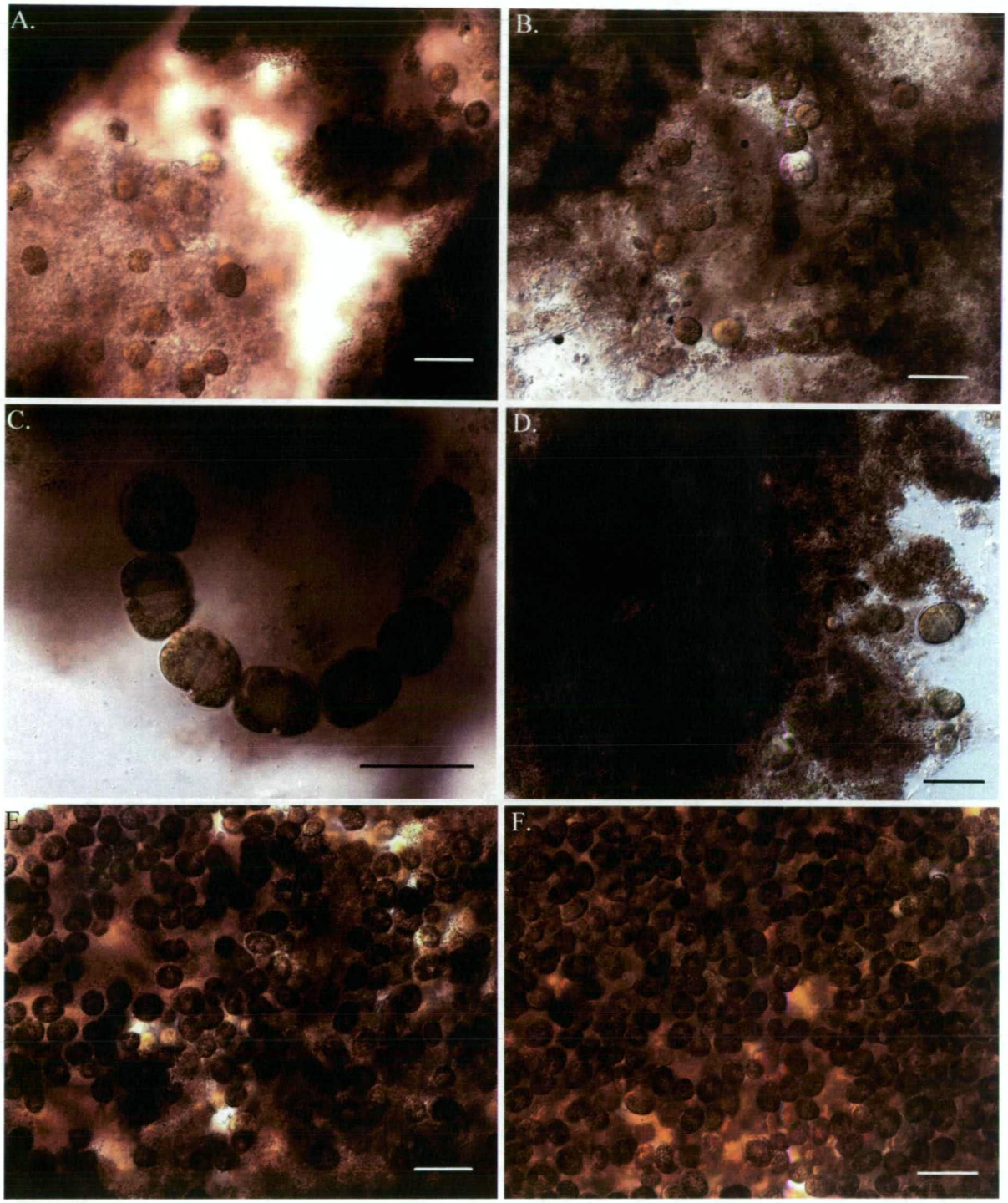

Fig. 4. Light microscope photographs of faecal material produced by Pacific oysters showing intact dinoflagellate cells after 24 h immersion in freshwater and 24 h depuration in filtered seawater (freshwater immersion experiment). (A) *Alexandrium catenella*-fed adult oyster: (B) *Alexandrium catenella*-fed spat oyster: (C) *Gymnodinum* catenatum-fed adult oyster: (D) *Gymnodinum catenatum*-fed spat oyster: (E) *Kryptoperidinium foliaceum*-fed adult oyster: (F) *Kryptoperidinium foliaceum*-fed spat oyster. Scale bars=50 μm

Chemical treatment of vegetative dinoflagellate cells and temporary cysts

Vegetative cells of the three dinoflagellate species could be killed at a concentration of 100 ppm hydrogen peroxide following an exposure period of 24 h when treated in filtered seawater. *Gymnodinium catenatum* and *K. foliaceum* cells were effectively eliminated at 50 ppm, yet at this concentration, up to 3% of *A. catenella* cells remained viable (Fig. 5). The chlorine dioxide biocide, BioSAFE 5, eliminated dinoflagellate vegetative cells at a concentration of 4 ppm. Three parts-per-million killed *G. catenatum* cells, whilst 4 ppm was required to destroy vegetative cells of *A. catenella* and *K. foliaceum* (Fig. 5).

Kryptoperidinium foliaceum temporary cysts were considerably more resistant to the chemical treatments compared to the fragile vegetative cells. A 200 ppm hydrogen peroxide concentration was needed to completely inactivate *K. foliaceum* temporary cysts following 24 h exposure (Table 5); and for BioSAFE 5, 20 ppm was required (Table 6). Figure 6 shows a *Kryptoperidinium foliaceum* temporary resting cyst exposed to 20 ppm of BioSAFE 5. The cyst wall and internal cell contents appear completely bleached.

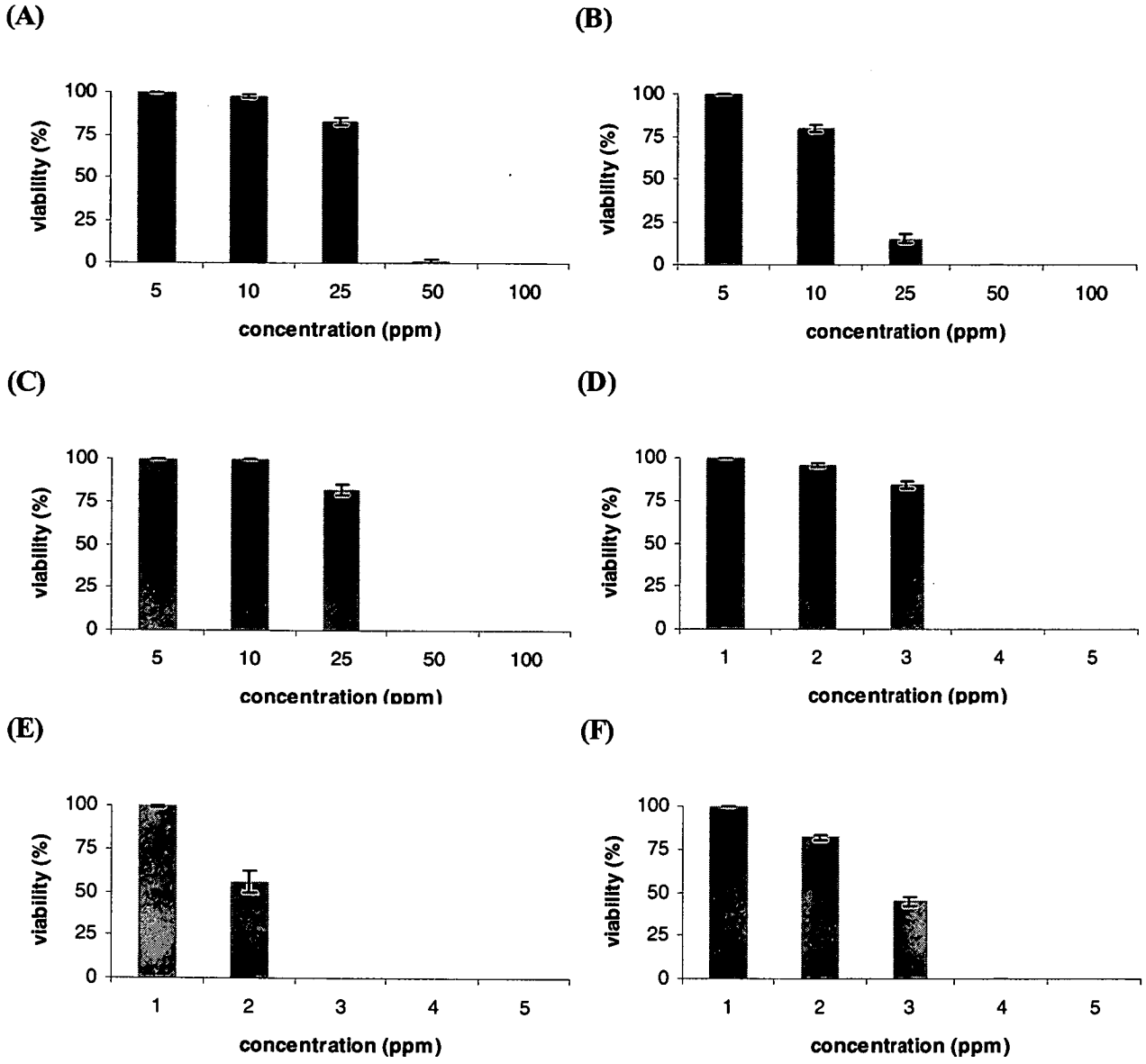

Fig. 5. Effect of varying concentrations of Hydrogen peroxide (A-C) and chlorine dioxide (D-F) on the viability of vegetative dinoflagellate species following 24 h exposure in filtered seawater. (A) *Alexandrium catenella*: (B) *Gymnodinium catenatum*: (C) *Kryptoperidinium foliaceum.* (D) *Alexandrium catenella*: (E) *Gymnodinium catenatum*: (F) *Kryptoperidinium foliaceum.* Bars indicate standard error.

Table 5. Germination success of temporary cysts of the dinoflagellate *K.foliaceum* after 24 h exposure to different concentrations of Hydrogen peroxide.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	72/90	117/184	82/119	251/393	63.9 ± 4.8
25	48/66	33/87	22/41	103/194	53.1 ± 10.1
50	29/70	71/202	52/91	152/363	41.9 ± 6.5*
100	35/67	50/91	67/79	152/237	64.1 ± 10.4
200	0/61	0/127	0/133	0/321	0*
400	0/104	0/78	0/221	0/403	0*

* Denotes significant difference compared to control (P<0.05).

Table 6. Germination success of temporary cysts of the dinoflagellate *K.foliaceum* after 24 h exposure to different concentrations of the chlorine dioxide biocide BioSAFE 5.

Concentration (ppm)	Viable cysts (viable/total)				Total cyst germination (%)
	Trial 1	Trial 2	Trial 3	Total	
0 (control)	47/51	38/43	77/101	162/195	83.1 ± 4.8
1	21/61	37/60	102/123	160/244	65.6 ± 14*
2	71/141	61/91	29/51	161/283	56.9 ± 4.9*
5	30/102	91/119	41/55	162/276	58.7 ± 15.4*
10	42/69	83/111	42/79	167/259	64.5 ± 6.3*
20	0/121	0/62	0/103	0/286	0*

* Denotes significant difference compared to control (P<0.05).

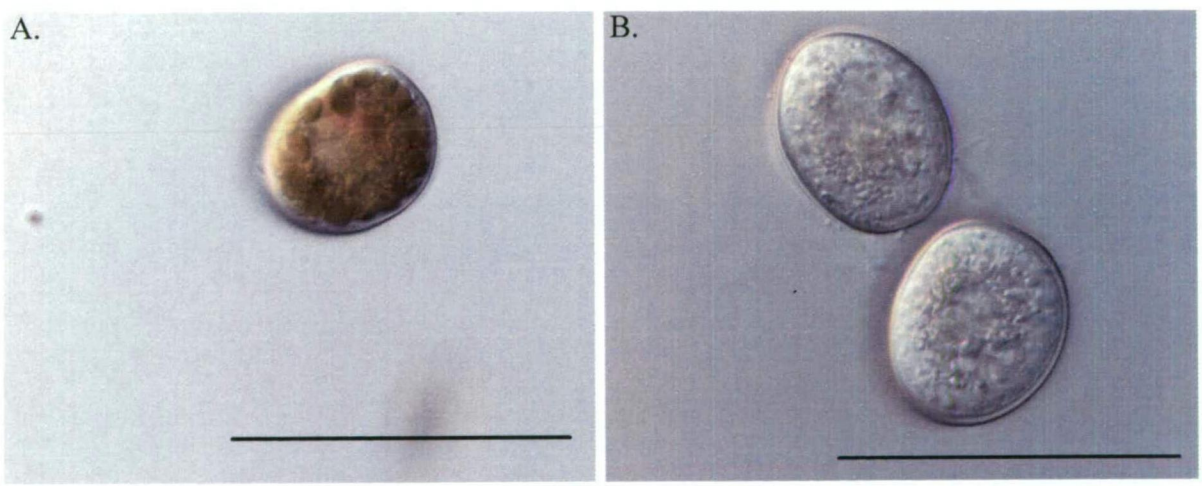

Fig. 6. Light micrograph of (A) Control *K. foliaceum* cyst: (B) *K. foliaceum* cysts exposed to 20 ppm of the chlorine dioxide biocide BioSAFE 5 for 24 h. Scale bars=50 μm

Chemical treatment of shellfish

When shellfish were exposed to hydrogen peroxide at the respective concentrations of 200 and 400 ppm for 24 h, 60 and 40% of the adult oysters that were fed *G. catenatum* produced faeces containing viable cells (Table 7). For the *A. catenella* and *K. foliaceum*-fed oysters, 100% of the trials contained viable cells when exposed to both hydrogen peroxide concentrations for 24 h (Table 7). Similar results were found when oyster spat was exposed to the hydrogen peroxide. *Gymnodinium catenatum* cells isolated from the spat faeces recovered in all the 200 ppm trials, whilst 80% of the 400 ppm spat trials produced faecal material containing viable cells (Table 7). All the spat trials that were fed *A. catenella* and *K. foliaceum* were found to produce faeces containing cells that could re-establish viable cultures following 24 h exposure to 200 and 400 ppm of hydrogen peroxide (Table 7).

When the exposure period was increased to 48 h, viable *G. catenatum* cells were not recovered from the 400 or 600 ppm spat treatments, however, the faecal material from several adult *C. gigas* exposed to 400 ppm contained viable cells (Table 7). Viable cells were found in the faeces of all of the *A. catenella* and *K. foliaceum*-fed adult oyster and spat trials, with the exception of the 400 ppm *A. catenella* trials (90%), after 48 h exposure to 400 and 600 ppm of hydrogen peroxide (Table 7). Figure 7 displays light micrographs of faecal material produced by Pacific oyster adults and spat showing intact dinoflagellate cells after 48 h exposure to 600 ppm hydrogen peroxide and 24 h depuration in filtered seawater.

Following 24 h exposure to the chlorine dioxide biocide, BioSAFE 5, 100% of the adult shellfish and spat trials that were fed *A. catenella* and *K. foliaceum* produced faeces containing viable cells following a dose of 20 and 40 ppm (Table 7). *Gymnodinium catenatum* cells recovered in 40 % of the adult oyster trials; and 80 and 100% of the spat trials when exposed to 20 and 40 ppm of BioSAFE 5, respectively, for a 24 h period (Table 7).

When the shellfish were exposed to BioSAFE 5 at concentrations of 40 and 60 ppm for 48 h, all the *K. foliaceum*-fed oysters produced faecal material containing viable cells (Table 7). Intact *A. catenella* cells that were capable of regrowth were

recovered from 60 and 80% of the 40 and 60 ppm trials, respectively, and all the spat trials were found to produce faecal material containing viable cells. Viable *G. catenatum* cells were excreted in 40% of the chlorine dioxide-treated adult oysters; whereas the faecal material collected from the spat trials contained no viable cells (Table 7). The presence of intact dinoflagellate cells in the shellfish faecal material produced after 48 h exposure to 60 ppm of BioSAFE 5 and 24 h depuration in filtered seawater is displayed in Figure 8. No shellfish mortalities occurred following 24 and 48 h exposure to 200-600 ppm of hydrogen peroxide or 20-60 ppm of chlorine dioxide.

Table 7. Summary of results from the chemical biocide treatment of Pacific oysters. Shows total numbers of adult oysters and spat trials that produced faecal material containing intact cells of the microalgal species (number of trials containing intact cells/total trials); and the total number of trials in which microalgal regrowth occurred from the shellfish faecal deposits (number of trials where regrowth of microalgal cells occurred/total trials). Faecal material was collected from each individual adult shellfish (5 trials) and spat trial (5 trials) after 24 h depuration in filtered seawater following 24 and 48 h exposure to the chemical biocides.

Chemical biocide	Concentration (ppm)	Adults						Spat					
		Alexandrium catenella		*Gymnodinium catenatum*		*Kryptoperidinium foliaceum*		*Alexandrium catenella*		*Gymnodinium catenatum*		*Kryptoperidinium foliaceum*	
		Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth	Intact	Regrowth
24h exposure													
Chlorine dioxide	20	5/5	5/5	4/5	2/5	5/5	5/5	5/5	5/5	5/5	4/5	5/5	5/5
	40	5/5	5/5	3/5	2/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Hydrogen peroxide	200	5/5	5/5	4/5	3/5	5/5	5/5	5/5	5/5	4/5	5/5	5/5	5/5
	400	5/5	5/5	3/5	2/5	5/5	5/5	5/5	5/5	5/5	4/5	5/5	5/5
48 h exposure													
Chlorine dioxide	40	4/5	3/5	2/5	2/5	5/5	5/5	5/5	5/5	0/5	0/5	5/5	5/5
	60	4/5	4/5	2/5	2/5	5/5	5/5	5/5	5/5	0/5	0/5	5/5	5/5
Hydrogen peroxide	400	5/5	4/5	3/5	2/5	5/5	5/5	5/5	5/5	0/5	0/5	5/5	5/5
	600	5/5	5/5	2/5	0/5	5/5	5/5	5/5	5/5	0/5	0/5	5/5	5/5

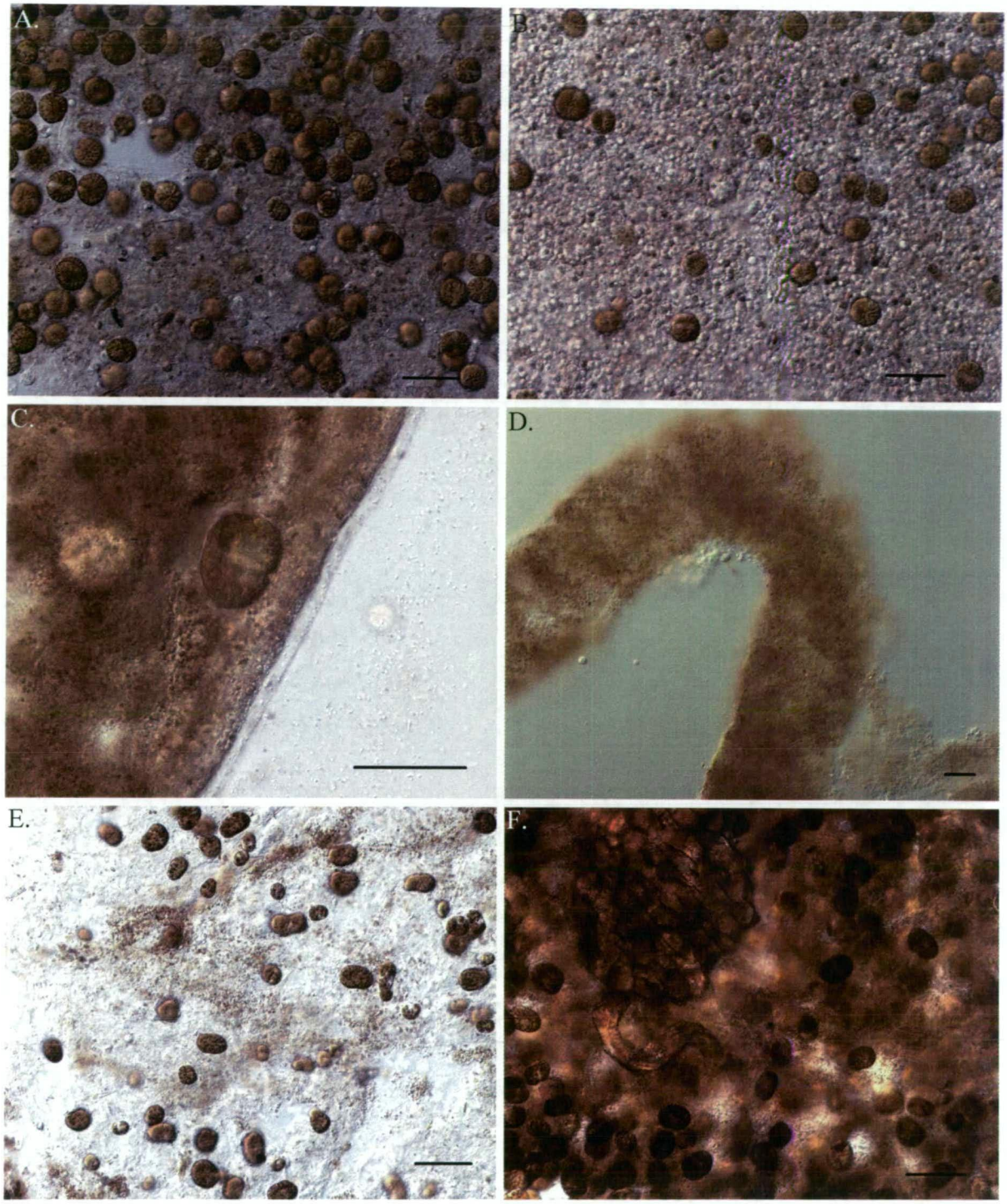

Fig. 7. Light micrographs of faecal material produced by Pacific oysters to show intact dinoflagellate cells after 48 h exposure to 600 ppm hydrogen peroxide and 24 h depuration in filtered seawater. (A) *Alexandrium catenella*-fed adult oyster: (B) *Alexandrium catenella*-fed spat oyster: (C) *Gymnodinum catenatum*-fed adult oyster: (D) *Gymnodinum catenatum*-fed spat oyster (no intact cells observed): (E) *Kryptoperidinium foliaceum*-fed adult oyster: (F) *Kryptoperidinium foliaceum*-fed spat oyster. Scale bars=50 μm

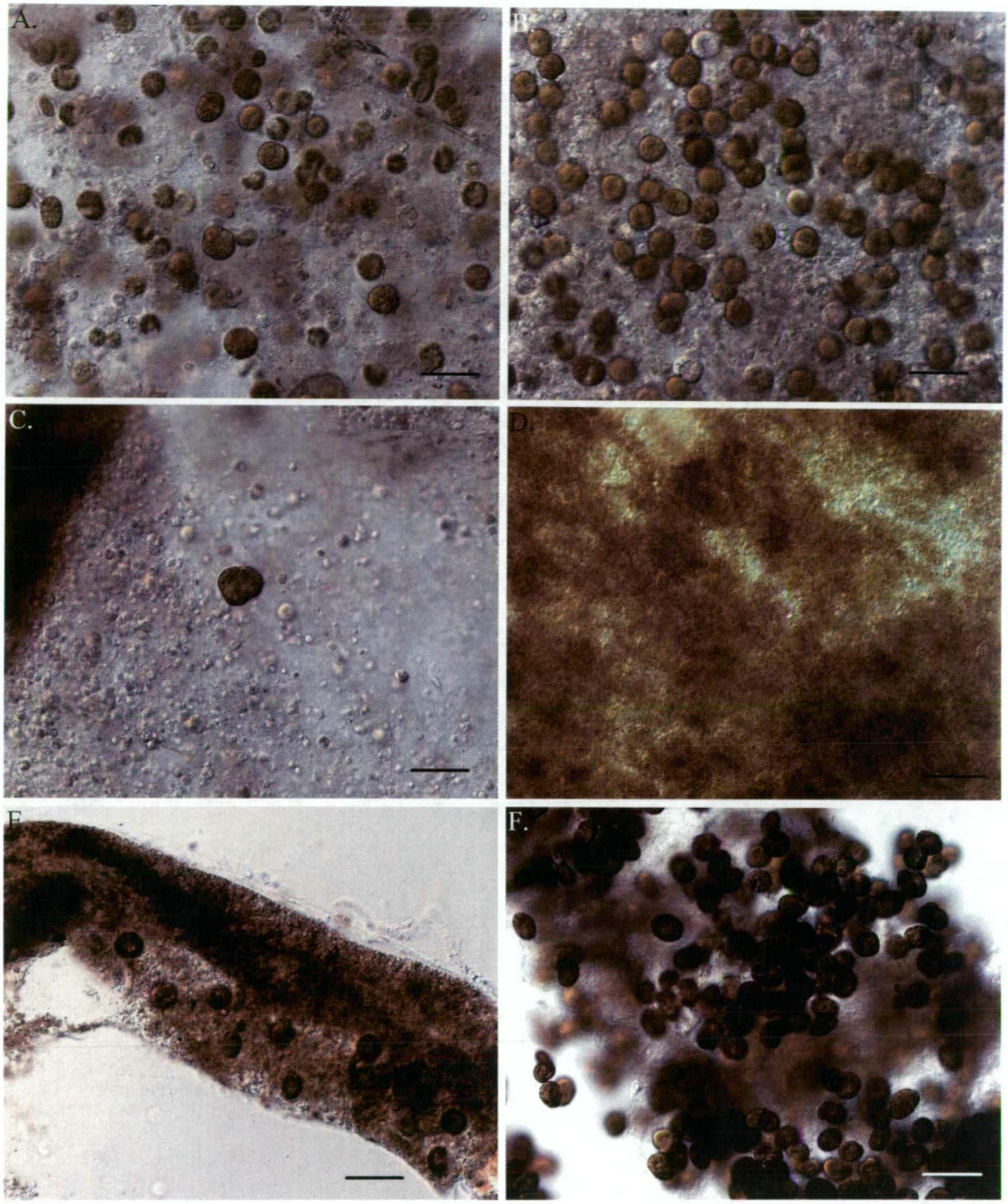

Fig. 8. Light micrographs of faecal material produced by Pacific oysters to show intact dinoflagellate cells after 48 h exposure to 60 ppm of chlorine dioxide and 24 h depuration in filtered seawater. (A) *Alexandrium catenella*-fed adult oyster: (B) *Alexandrium catenella*-fed spat oyster: (C) *Gymnodinum catenatum*-fed adult oyster: (D) *Gymnodinum catenatum*-fed spat oyster (no intact cells observed): (E) *Kryptoperidinium foliaceum*-fed adult oyster: (F) *Kryptoperidinium foliaceum*-fed spat oyster. Scale bars=50 μm

The *K. foliaceum*-fed oysters that were exposed to extremely high concentrations of the chemical biocides still produced faeces containing viable cells following an exposure period of 96 h (Fig. 9). One to three individual shellfish were killed in the 200 ppm chlorine dioxide treatment of adult oysters and in the adult and spat hydrogen peroxide treatments (Table 8). Nonetheless, in most cases, the surviving shellfish produced faecal material containing viable *K. foliaceum* cells. Viable *K. foliaceum* cells were recovered from all the spat oyster trials following 96 h exposure to high concentrations of hydrogen peroxide (2000 and 4000 ppm) and BioSAFE 5 (200 and 400 ppm) (Table 8). All the surviving adult oysters in the BioSAFE 5 treatments produced faecal material containing intact *K. foliaceum* cells, and indeed many of these cells were able to recover to form growing populations. Viable *K. foliaceum* cells were also recovered from the faeces in 3 of the 4 live adult oysters in the 2000 ppm hydrogen peroxide treatment; and 2 of 3 of the live specimens in the 4000 ppm trials (Table 8).

Table 8. Summary of results of the chemical biocide treatment of *Kryptoperidinium foliaceum*-fed Pacific oysters (high concentration experiment). Shows total numbers of adult oysters and spat trials that produced faecal material containing intact cells of the microalgal species (number of trials containing intact cells/total trials); and the total number of trials in which microalgal regrowth occurred from the shellfish faecal deposits (number of trials where regrowth of microalgal cells occurred/total trials). Faecal material was collected from each individual adult shellfish (5 trials) and spat trial (2 trials) after 24 h depuration in filtered seawater following 96 h exposure to the chemical biocides.

Chemical biocide	Concentration (ppm)	Adults		Spat	
		Intact	Regrowth	Intact	Regrowth
Chlorine dioxide	200	3/5 (2)	3/5 (2)	2/2	2/2
	400	5/5	5/5	2/2	2/2
Hydrogen peroxide	2000	4/5 (1)	3/5 (1)	2/2 (3)	2/2 (3)
	4000	3/5 (2)	2/5 (2)	2/2 (2)	2/2 (2)

The numbers in parentheses are the number of individual shellfish killed.

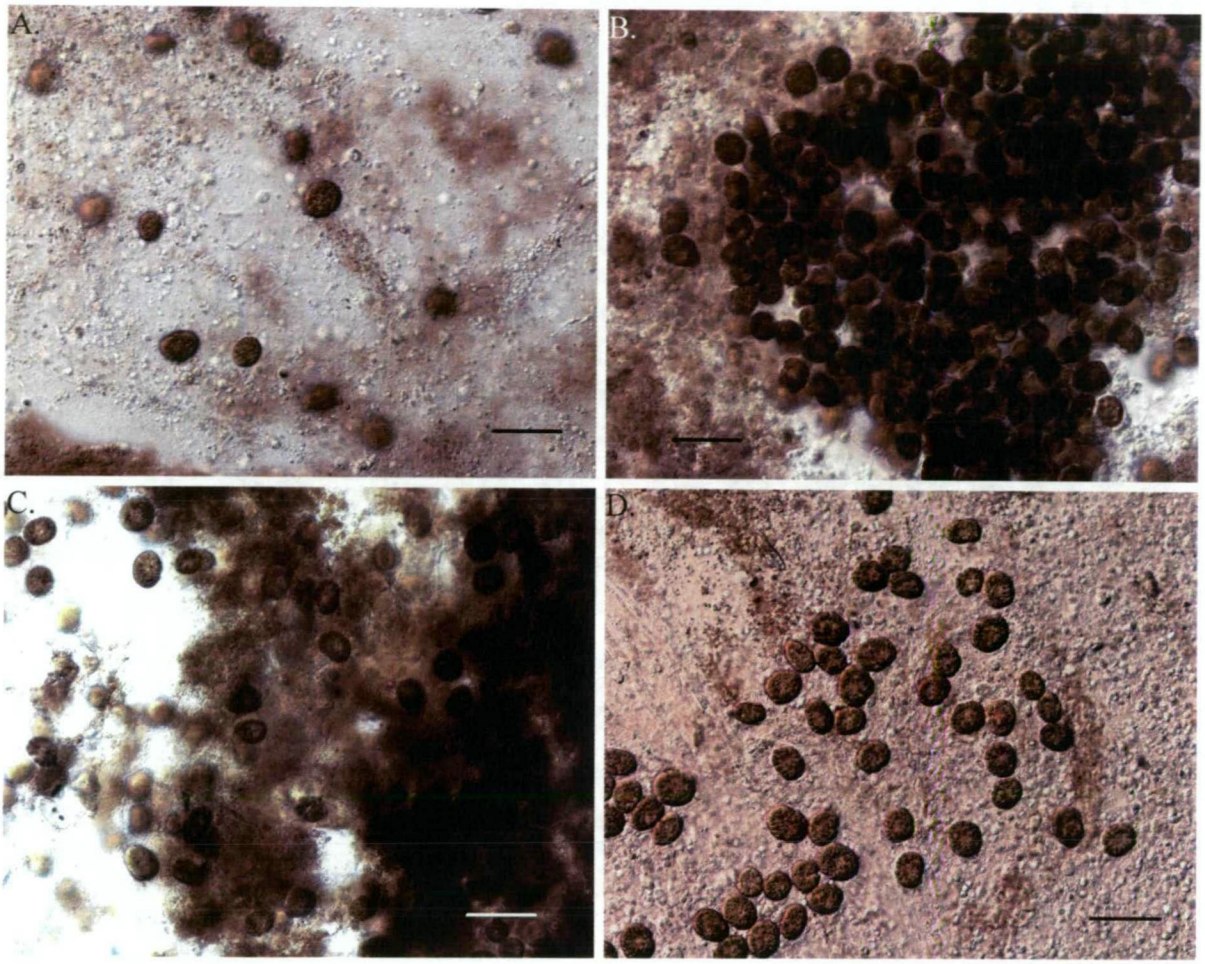

Fig. 9. Light micrographs of faecal material produced by *Kryptoperidinium foliaceum*-fed Pacific oysters to show intact cells after 96 h exposure to 400 ppm of chlorine dioxide and 4000 ppm of hydrogen peroxide. (A) Chlorine dioxide-treated adult oyster: (B) Chlorine dioxide-treated spat oyster: (C) Hydrogen peroxide-treated adult oyster: (D) Hydrogen peroxide-treated spat oyster. Scale bars=50 μm

8.5 Discussion

Since the 1990's it has been widely recognised that some microalgal species can survive gut transit in Pacific oysters and are therefore potential vectors for the introduction of toxic or nuisance phytoplankton to new areas when shellfish are moved for outgrowing purposes or to establish new fisheries. This study is the first to test the effectiveness of potential treatment options for eliminating marine microalgae following their ingestion by Pacific oysters. Treatment options currently being considered or used by shellfish farmers for the control of unwanted organisms include freshwater immersion, chemical treatment and seawater depuration systems;

however, until now, the extent to which these techniques could reduce this risk of microalgal introductions was unknown. In the present experiment, dinoflagellate cells ingested by both adult and spat Pacific oysters could not be eliminated by either immersing the shellfish in freshwater or by chemical treatment with hydrogen peroxide and chlorine dioxide. Depuration in filtered seawater was identified as the only treatment option that could effectively eliminate dinoflagellate cells from the digestive tract of the shellfish.

Several researchers have suggested that seawater depuration systems may provide an effective means of purging bivalve shellfish of harmful microalgae. Scarratt *et al.* (1993) suggest that holding transferred mussel and scallop spat in UV irradiated, recirculating seawater for a period of 12 hours would be sufficient to purge the shellfish of *Alexandrium tamarense* cells. Similarly, Hégaret *et al.* (2008) exposed seven species of bivalve molluscs (including eastern oysters *Crassostrea virginica*) to several strains of harmful microalgae including *Prorocentrum minimum, Alexandrium fundyense, Heterosigma akashiwo, Aureococcus anophagefferens, Karenia mikimoto* and *Alexandrium monilatum*, and concluded that a 24 to 48 hour depuration period in seawater may minimise the risk of introducing toxic phytoplankton to new areas via shellfish translocations. In the present study, a depuration period of 7 days in filtered seawater was required for the complete elimination of dinoflagellate cells from the digestive tract of *C. gigas*, and the required depuration period did not differ between adult and spat oysters. This period of depuration is considerably longer than those suggested by Scarratt *et al.* (1993) and Hégaret *et al.* (2008), however it should be noted that gut retention time in bivalves is highly variable. Shellfish kept under different conditions or collected at different time of the year could have different gut retention times (Scarratt *et al.*, 1993).

In the present work, no viable *Gymnodinium catenatum* cells were recovered from the oyster faeces after 4 days of depuration in filtered seawater, whereas the oysters that were fed *Alexandrium catenella* and *Kryptoperidinium foliaceum* produced faecal material containing viable temporary cysts after 6 days. These findings indicate that the chemical, physical and mechanical constraints the dinoflagellates are exposed to during gut transit in the Pacific oyster act to destroy vegetative cells after

4 days; however this also acts to promote the formation of temporary cysts, which cannot be destroyed inside the gut and must either be flushed out using depuration procedures or eliminated using another treatment method.

The lengthy depuration period required for the complete elimination of microalgae from the digestive tract of Pacific oysters identified in the present study (7 days) is unlikely to be compatible with current oyster farming practises and may impact on the health or condition of the shellfish. One alternative may be to hold translocated shellfish in a closed seawater system and feed the shellfish non-toxic microalgae, such as the green-flagellate *Tetraselmis*, until no other species are retained in the digestive tract.

A number of previous studies have assessed the effectiveness of freshwater immersion as a control option for fouling organisms and other pest species that adhere to the exterior surface of shellfish (Park *et al.,* 1998; Forrest and Blakemore, 2006); however no data are available on the effect of freshwater treatment on organisms contained in the mantle cavity or digestive tract of shellfish. Forrest *et al.,* (2006) found that *Undaria pinnatifida* gametophytes could be completely eliminated following 1-2 days immersion in freshwater, yet such treatment did not prevent the introduction of many invertebrate and seaweed species associated with Pacific oyster imports from Japan to Europe during the 1970's (Mineur *et al.,* 2007). In the current experiment, immersing Pacific oysters in freshwater for a period of 24 hours failed to eliminate dinoflagellate cells ingested by the Pacific oyster.

In Australia, Tasmania and South Australia are the major Pacific oyster growing states. Presently, a considerable amount of oyster spat is exported from Tasmania to South Australia (Love and Langenkamp, 2003), however concerns exist over this importation as Tasmanian waters contain several pest species (including *Asterias amurensis, Undaria pinnatifida* and *Boccardia knoxii*) which if introduced to South Australia could result in significant ecological and economic problems. The current policy on the export of oyster spat from Tasmania to South Australia requires shellfish to be treated with freshwater for a period of 12 hours followed by 12 hours depuration in sterilised seawater prior to translocation (PIRSA, 2001). In the present study, after the initial 24 hour freshwater immersion period, viable microalgal cells

were still present in *C. gigas* faecal material produced after an additional 6 days of depuration in filtered seawater. Given this, the current protocol adopted by Tasmanian oyster spat suppliers is unlikely to reduce the risk of introducing harmful microalgae into South Australia with the translocation of live oyster spat consignments.

Oyster farmers are currently considering using chlorine for the control of potentially invasive species such as *Undaria pinnatifida, Asterias amurensis* and *Carcinus maenas.* Chlorine has been used in the past to sterilise effluent from oyster processing plants in New South Wales (NSW) due to concerns about the possibility of transferring toxic dinoflagellates from Tasmanian waters to NSW. While it is likely that organisms occurring on the exterior surface of oysters could be controlled using chemical treatments, doubts exist over their effectiveness against organisms contained inside the shellfish. In the present work, although vegetative dinoflagellate cells and temporary cysts were killed at concentrations of 200 ppm of hydrogen peroxide and 20 ppm of BioSAFE 5 following 24 hours exposure when treated in filtered seawater, hydrogen peroxide concentrations of 200-600 ppm, and 20-60 ppm of the chlorine dioxide biocide, BioSAFE 5, could not eliminate dinoflagellate cells of the three test species in the digestive tract of Pacific oysters after 24 and 48 h exposure. No viable *G. catenatum* cells were recovered from the faecal material of the spat oysters following 48 h exposure to hydrogen peroxide and BioSAFE 5, however, this mortality is likely due to the lack of light or the digestive processes of the shellfish rather than exposure to the chemical biocides.

Very high chemical biocide concentrations failed to kill ingested *K. foliaceum* cells following an exposure period of 96 h. Viable temporary cysts were recovered from faecal material collected from adult and spat oysters that were exposed to hydrogen peroxide at concentrations of 2000 and 4000 ppm; and 200 and 400 ppm of chlorine dioxide. Several shellfish were killed following 96 h exposure to these concentrations; however it is unclear whether mortality was due to the toxicity of the chemical biocides or the extended period of complete shell closure.

The results of the chemical treatment experiments indicate that ingested microalgal cells are likely to prove difficult to kill with chemical biocides. It would appear that

Pacific oysters are able to detect the presence of toxicants in the water as the shellfish were observed to completely close their shells and cease filtration. A similar reaction was reported by Borcherding (1992), who examined valve movements in the European zebra mussel (*Dreissena polymorpha*) in response to toxic discharges in the aquatic environment. Upon detecting the presence of toxic substances, the zebra mussels were found to rapidly close their shell, after which they periodically opened their valves to test the suitability of the water, and only switched to normal filtration when conditions were acceptable. The demonstrated ability of *C. gigas* to endure extremely high doses of hydrogen peroxide (4000 ppm) and chlorine dioxide (400 ppm) does, however, indicate that the use of these chemicals may be an effective means of eradicating organisms that occur on the outside shell surface of the bivalve species and may provide alternative, more environmentally sound treatment options than chlorination for the sterilisation of effluent from seawater depuration facilities or shellfish processing plants.

Heat treatment was not tested during the present study; however this method is commonly used to remove fouling organisms and pest species from oysters and other bivalve shellfish. Immersion of shellfish for 5-15 seconds in 55-60°C seawater effectively eradicates a range of organisms including *Undaria pinnatifida* and *Mytilus* sp. (Park *et al.,* 1998; Forrest and Blakemore, 2006). Immersion for shorter periods (3 s) at higher temperatures (80°C) has been used to remove fouling organisms and macroalgal species from *C. gigas* (Minuer *et al.,* 2007), yet such short exposure periods are likely to have a negligible impact on microalgal species contained in the digestive tract of Pacific oysters. During *G. catenatum* blooms in New Zealand, translocated Pacific oyster spat was shown to endure a 4 minute 'spa bath' in 50°C water which was used to eliminate dinoflagellate cysts on the outside of the shell. Most vegetative microalgal cells are readily killed at temperatures as low as 35°C with exposure times between 30 mins and 5 hrs and *G. catenatum* resting cysts are effectively inactivated at exposures ranging from 1 h at 37.5°C, to 2 min at 38-40°C, and 30 seconds at temperatures of 44.5-46.3°C (Bolch and Hallegraeff, 1993; Hallegraeff *et al.,* 1997). *Crassostrea gigas* has a broad temperature tolerance, with a range of -1.8 to 35°C (FAO, 2006), and thermotolerance can be increased to 37-39°C by exposing shellfish to increased but sub-lethal temperatures (<41°C) for 1

h (Rajagopal *et al.*, 2005). Given this, immersing Pacific oysters in seawater at temperatures of >35-39°C for periods of 30 mins to several hours may effectively eliminate ingested microalgal cells, however it must be determined whether lethal temperatures can be achieved inside the digestive tract and mantle cavity of the shellfish without causing significant mortalities or negative impacts on the heath or quality of the oysters. The effect of hot water, and even very cold water treatment on the viability of microalgal cells following their ingestion by Pacific oyster requires further investigation.

In summary, many of the treatment techniques currently used by oyster farmers for controlling unwanted organisms do not significantly reduce the risk of introducing harmful microalgae to new areas when shellfish are translocated for outgrowing purposes or to establish new fisheries. The elimination of dinoflagellate cells following ingestion by Pacific oysters cannot be achieved using chemical biocides or by freshwater immersion. Depuration in filtered seawater for a period of 7 days was identified as the only treatment option capable of purging *Crassostrea gigas* of viable dinoflagellate cells and this method is likely to be incompatible with current oyster farming practices and may impact on the health or quality of the shellfish. A possible alternative would be to maintain the transferred shellfish on a non-toxic microalgal diet in a closed filtered or UV-irradiated seawater system until no other species are retained in the digestive tract. Effluent from these treatments systems could be sterilised with hydrogen peroxide or chlorine dioxide. Further research should investigate alternative or more convenient treatment options to minimise the risk of toxic microalgal introductions through the relocation of Pacific oysters. Until an effective treatment option is discovered, the geographic transfer of Pacific oyster stocks should be regulated to minimise such risks.

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